VOLUME 44 NUMBER 5, PART II NOVEMBER, 1957

# AMERICAN JOURNAL OF OPHTHALMOLOGY

THIRD SERIES FOUNDED BY EDWARD JACKSON

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## The First JONAS S. FRIEDENWALD MEMORIAL LECTURE

(Delivered by John E. Harris, M.D.)

## PROCEEDINGS

of the

Association for Research in Ophthalmology, Inc.

Twenty-sixth Meeting

New York, New York

June 3, 4, 5, and 6, 1957

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*Subscription price in United States twelve dollars yearly. In Canada and foreign countries fourteen dollars.* Published monthly by the Ophthalmic Publishing Company. Subscription and Advertising Office: 664 North Michigan Avenue, Chicago 11, Illinois. Entered as second class matter at the post office at Menasha, Wisconsin. Printed in U.S.A.

AMERICAN JOURNAL OF OPHTHALMOLOGY

SERIES 3 · VOLUME 44 · NUMBER 5 · PART II · NOVEMBER, 1957

and the

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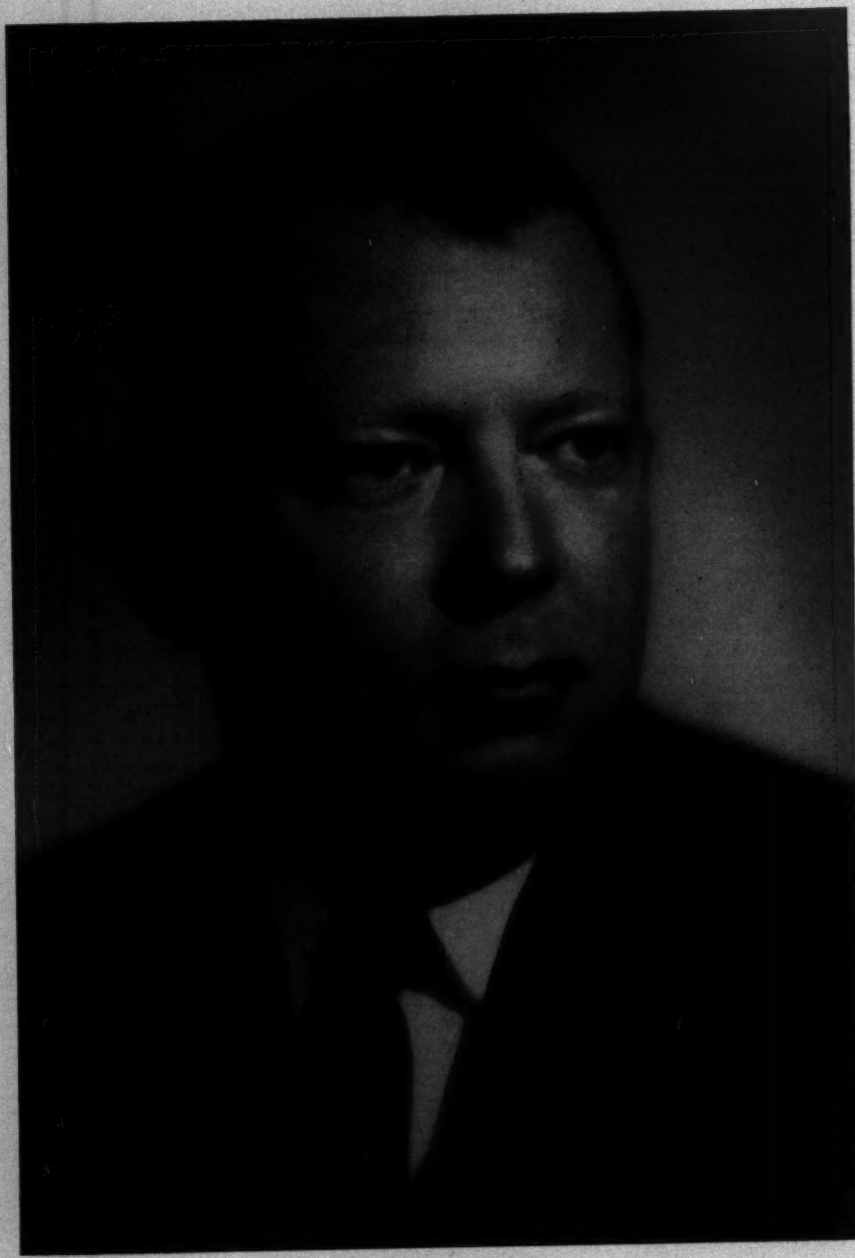

JOHN E. HARRIS, M.D.

# REMARKS ON ACCEPTANCE OF FRIEDENWALD MEMORIAL PLAQUE

June, 1957

JOHN E. HARRIS, M.D.

Of his generation Dr. Jonas Friedenwald was certainly one of the foremost, if not the foremost, experimentalist devoted to the study of the problems of ophthalmology. It is particularly appropriate, therefore, that the Association for Research in Ophthalmology should dedicate part of its meeting to his memory. For the privilege of presenting the Friedenwald Memorial Lecture I am very grateful.

My personal contacts with Dr. Friedenwald were all too few and I can add nothing to the many tributes already written by others, tributes which have attested to his scientific acumen, his widespread interests, and his devotion to his fellow man. Instead I should like to discuss briefly my reaction to his impact on the field of ophthalmology, a field with which I first became acquainted when I entered medical school in 1946.

From the time I first read his published papers I have been intrigued by, and marveled at the extreme imagination with which he approached his experimental work. He brought an entirely fresh outlook to every problem. Consider his studies of aqueous formation which first began to bear fruit some 20 years ago. Physical chemistry as applied to biology came into its own in the 1920's. The popular explanation for biologic phenomena was in terms of simple (or complicated as the case may be) equilibria. The issue of aqueous formation seemed to have been settled by chemical analyses and the Donnan equilibrium—to all except Jonas Friedenwald. Perhaps, intuition led him to disbelieve this thesis. At any rate he recognized that the problem would have to be reopened from an entirely different perspective whereupon he undertook an amazing series of experiments which continued for many years. Indeed, this work and the resultant formulation of his ideas serve as a point of departure in present-day thinking on this subject. Similar ingenuity and radical departure from a stereotyped approach marked his other endeavors. Coupled with his imagination was an experimental audacity which saw him shrink from no task, however tedious or impossible it appeared.

To channel properly this imagination required an ability to apply various disciplines, particularly the basic science disciplines, to problems of his interest, an interest that was extremely diversified. This amalgamation of disciplines was well exemplified in Dr. Friedenwald's approach to pathology. One could say, as a matter of fact, that he was the first experimental pathologist in ophthalmology. He was not satisfied with the purely morphologic interpretation but rather was interested in the fundamental alteration of the physiology of the cell which caused the disease process presented to the clinician or the microscopist.

By such an approach Jonas Friedenwald succeeded in traversing the barriers of the various disciplines. He employed the basic science techniques for the study of the normal and diseased eye and in return contributed to those fields by applying the lessons learned from ocular tissues. In so doing, Dr. Friedenwald maintained constant and penetrating contact with other efforts in clinical and experimental medicine.

This example, I feel, is one which Jonas Friedenwald would not have us forget. The breakdown of communication between the various specialties is not an overplayed theme. One of the worst offenders is the ophthalmologist who with his rather peculiar jargon has succeeded unwittingly in isolating himself from his medical confreres. The same criticism applies to many of us who work in laboratories devoted to research on problems of the eye. We borrow the tools

we need from the other disciplines but often fail to return the favor when we do not apply the lessons learned from the eye to the solution of community problems.

Our responsibility to the education of the medical student deserves special mention. It is becoming increasingly apparent that seemingly diverse diseases affecting individual systems represent a common biochemical or physiologic fault at the cellular level. Undergraduate instruction gains considerably when ophthalmology is approached from this basic point of view. Moreover, the ophthalmologist can offer the medical student an insight into many aspects of sensory physiology, certain phases of vegetative physiology and biochemistry and an excellent approach to pharmacology. In short he is in a good position to help the student integrate the preclinical and clinical years. The future of ophthalmology as an undergraduate subject is assured when its teaching is approached from this point of view.

Thus, I interpret some of the lessons which Dr. Friedenwald has left us. He would integrate ours with other medical specialties and resist any attempt to make ophthalmology a superspecialty (surgical or otherwise) beyond the ken of the average physician. The emphasis of clinical practice in ophthalmology must be built on a sound understanding of basic principles of physiology and biochemistry and their pathologic alterations. I submit that Jonas Friedenwald would approve.

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## BIOGRAPHICAL DATA

### John E. Harris, M.D.

John E. Harris was born in Toledo, Ohio, on December 27, 1913. He received his elementary and secondary education in that city and graduated from the University of Toledo in 1935 with a major in chemistry. He subsequently entered graduate school at the State University of Iowa where he received his Ph.D. in biochemistry in 1940. After remaining a year on the staff as a research associate he went to the University of Pennsylvania as a National Research Council Fellow to work with Dr. M. H. Jacobs of the Department of Physiology. During these years his investigative work was concerned largely with the permeability characteristics and the factors controlling the cation distribution of the erythrocyte. In connection with this work he developed techniques which he subsequently adapted to the study of the lens and cornea.

In 1942, he entered the U. S. Army Air Corps as an aviation physiologist. Upon discharge from the service in 1946, he decided to fulfill a life-long ambition to obtain his medical degree. An opportunity to achieve this goal was offered by Dr. Kenneth C. Swan, head of the Department of Ophthalmology, University of Oregon Medical School, a personal friend with whom Dr. Harris had become acquainted while at the State University of Iowa when Dr. Swan was a resident in ophthalmology.

With Dr. Swan's encouragement and help, Dr. Harris entered the University of Oregon Medical School and became a research associate in the Department of Ophthalmology in the fall of 1946. From this beginning has grown a warm and rewarding relationship which continues to this day.

Graduating from the University of Oregon Medical School in 1950, Dr. Harris interned at the Walter Reed Army Hospital. In 1951 he returned to the University of Oregon Medical School as an assistant professor of ophthalmology and director of the John E. Weeks Memorial Laboratory. That year he received the five-year appointment as a Markle Scholar in Medical Science. In

1954, he became an associate professor of ophthalmology and joined the staff of the Department of Biochemistry as a research associate.

One of John Harris' major interests has been medical education. Integrating the teaching of ophthalmology with the basic science disciplines has been a particular goal. At the same time through staff conferences he has tried to present a common point of reference to other clinical specialties.

In addition he has found time to continue a comprehensive program of clinical and basic laboratory investigation. While biochemistry of the eye remains his first love, his basic research has extended into pharmacology and related fields. A noteworthy aspect of his laboratory research program has been a desire to stimulate an interest and provide training in basic ophthalmological research for promising young people. Several have worked with him while obtaining their medical education and/or graduate degree in biochemistry. Most of his nearly 30 publications on ophthalmologic subjects have been in association with these younger colleagues.

In 1938, Dr. Harris was married to Bessie Hatherly, the two having met in undergraduate school and having attended graduate school at the State University of Iowa together. Mrs. Harris has been of invaluable assistance in various academic efforts undertaken by Dr. Harris.

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# THE PHYSIOLOGIC CONTROL OF CORNEAL HYDRATION*

## The First Jonas S. Friedenwald Memorial Lecture

John E. Harris, M.D.
*Portland, Oregon*

It is well known that when a piece of cornea is placed in water, normal saline or even hypertonic solution it swells to many times its normal weight, the increase in volume being largely due to an imbibition of the bathing fluid. (The cornea is rather unique in this regard. Other collagen tissues such as cartilage also swell from the dry state but the degree of swelling is generally less than that of the cornea.[1]) All the evidence that can be brought to bear on the problem, both clinical and laboratory, indicates that the corneal swelling potential is present in the normal eye in vivo. Some restraining force then must act to maintain the hydration. In recent years it has become apparent that this force is an active process, that is, it requires the mediation of the living cell.

Time does not permit an exhaustive review of the literature prior to the time that such a thesis became essential. Much of this can be found in the excellent summation by Cogan and Kinsey.[2] In brief, predominate opinion emphasized the necessity for considering the endothelial and epithelial barriers impervious to bulk aqueous and tears. As Duke-Elder[3] stated, "Both the epithelium and the endothelium are physiologically waterproof," a truism which can still be applied today. Such a connotation would allow for an exchange of water which was in fact demonstrated by Cogan and Kinsey[4] using deuterium oxide. It would require, however, that the barriers either be impermeable to the major ions of the bathing fluids or that some active process be operative. Until fairly recently the data indicated that the limiting barriers were impermeable to sodium chloride and supported the hypothesis that normal

* From the John E. Weeks Memorial Laboratory, Department of Ophthalmology, University of Oregon Medical School. Except for the studies with hyaluronic acid sulfate, the experimental studies were supported by Grant B-187 of the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda 14, Maryland.

corneal turgescence was maintained by an osmotic gradient applied across the epithelial and endothelial surfaces by aqueous and tears hyperosmotic with respect to the plasma which filtered into the stroma at the limbal junction.[2]

The series of papers of Potts and his associates[5] and the work of Maurice,[6] both of whom employed tracer ions, indicated that the barriers were indeed permeable to sodium. Since the possibility of a selective impermeability to some major anion, such as chloride, seemed extremely unlikely it was necessary to conclude that an active process normally "pumped" fluid from the cornea.

This general concept gives no clue as to the particular substance actively extruded from the cornea. It could be an anion, a cation, water itself, or a combination. In any event, the movement would require active metabolic mediation and would, therefore, be dependent upon normal corneal metabolism. That such was the case was soon proved, or in certain respects definitely reaffirmed, during the past four to five years in many laboratories. It is these more recent studies that I would like to review, using our own as point of departure for this discussion because they employ a technique which in many respects gives the most unequivocal evidence for such an active movement.

Parenthetically, it is interesting to note that a parallel path has been followed by those who have studied electrolyte and water balance of the cell. Early workers were unable to demonstrate a movement of cations across cell membranes and considered them to be impermeable to the positive ions. Later, however, it became abundantly evident that an exchange of both sodium and potassium occurred across the cell barrier and the existence of a cation "pump" capable of maintaining normal cation relationship was subsequently demonstrated.[7] It is still not clear, however, that this cation "pump" alone maintains cellular water at its normal level and that a water "pump" is not essential in addition.[8–10]

## Corneal cation and water alterations induced by temperature change

Many cells, when placed in the cold, tend to assume ionic equilibrium with their fluid environment, losing their characteristically high potassium concentration and gaining in sodium. This exchange was considered to be due to an alteration in membrane permeability until it was shown that the cation shift could be reversed, that is, the potassium driven into the cell and sodium from it, when the cell was subsequently replaced at body temperature.[11] Presumably the shift in cations induced by cold resulted from a decrease in metabolic intensity thereby reducing substantially to zero the active transport of cations which normally maintained the high potassium and low sodium concentrations. With the reestablishment of the normal temperature active transport of cations was resumed. This temperature reversal technique has been employed for the study of factors controlling the cation balance and hydration of the erythrocyte,[11, 12] lens,[13] retina[14] and other cellular structures.

The cation distribution in the cornea is quite the reverse of that seen in cells. Our analyses of 93 rabbit corneas have shown the average sodium concentration to be 154 mEq/1,000 gm. of water and the potassium to be 29.3 mEq/1,000 gm. of water. The corneal cation values then generally resemble those of plasma and extracellular fluid, except for the fairly high potassium level which, of course, reflects the cellular content of the cornea. In this respect, therefore, it is a connective tissue. Nevertheless, it seemed reasonable to suppose that a temperature reversible hydration could be demonstrated in the cornea and that the technique would prove useful for determining with some finality whether the metabolism of the cornea played a significant role in the maintenance of corneal hydration and possibly indicate whether water or some ion or both was being actively extruded from the cornea. This indeed has proved to be so[15–18] and certain of

which follows is a narrative account of our already published work employing this technique.

The first task in applying this technique was to find an experimental situation in which the corneal water and ionic composition could be maintained during incubation in vitro at body temperature. The use of excised corneal pieces immersed in an appropriate medium was not possible since these swell readily in isotonic solution at 37°C. When the anterior portion of the globe, devoid of intraocular contents, was immersed in solution at 37°C., the uptake of water and sodium was considerably reduced but nonetheless appreciable.[16] It was noted, however, that the water and sodium contents of this preparation tended to stabilize after a short period of incubation and then remain fairly constant for a number of hours. Similar changes were observed when the entire eye was immersed in solution. The results of Langham and Taylor[19] are comparable. It seems likely that the initial rise observed in these preparations where the scleral-corneal junction is not interrupted is due to the movement of sodium and water across the subconjunctival and episcleral space. A new steady-state is then established which is maintained for a number of hours by an active "pump" process.

When the isolated eye was placed in a moist chamber at 37°C. the cation and water contents of the cornea could be maintained at normal values for at least 12 hours.[16] (It is interesting to note that the glucose of the aqueous is reduced to substantially zero after six hours when the isolated eye is incubated at 37°C. in a moist chamber. Residual glucose, or metabolic intermediates remaining in the cornea must provide the needed energy for the maintenance of corneal hydration at normal levels for the next six hours.)

Because of the above we have preferred to employ the moist chamber rather than the immersion technique in our studies of the factors effecting corneal hydration.

Refrigeration of the isolated rabbit eye in a moist chamber at 0°C. resulted in a fairly uniform increase in hydration.[16] The water content tended to reach a maximum of a little greater than twice the normal value at approximately 120 hours. At the same time the sodium and potassium *contents* (mEq/100 gm. dry wt.) increased. The net effect, however, was such that a decrease in the cation *concentration* (mEq/1,000 gm. of water) occurred.

Greater hydration can be observed during refrigeration if the cornea is treated with strong detergents or if the endothelial barrier is mechanically disturbed. Thus the level reached in the unaltered eye represents a new steady-state rather than some physical or chemical limitation imposed by a limited aqueous supply.

The question arose whether this dilution of corneal cations in the cold could be attributed to the movement into the cornea of fluid of the composition of aqueous or whether a preferential movement of water had occurred.[16] The problem was complicated by the fact that during refrigeration the cation values of the aqueous did not remain constant; rather, the sodium concentration fell and the potassium rose. The total base, however, remained essentially unaltered. Calculations showed that if the total increase in hydration had resulted from the movement of fluid with the composition of aqueous the concentration of total base in the cornea should have fallen to 175 mEq/1,000 gm. of water after 20 hours refrigeration. Actually, the concentration fell to 165 mEq/1,000 gm. of water. Consequently, a preferential movement of water must have occurred—amounting to about 20 percent of the total hydration. After 20 hours the hydration could be attributed to the movement of fluid having the composition of aqueous. The preferential movement of water under these conditions was compatible with the view that the cornea is normally hyperosmotic with respect to aqueous.

When the eyes so refrigerated were subsequently incubated at 37°C. for six hours the

corneal water content was reduced to substantially the in vivo value. At the same time a migration of sodium from the cornea was observed, the potassium *content* remaining fairly constant. In spite of this loss of sodium there was an initial rise (within an hour) in sodium *concentration* to the approximate value of fresh tissue, a level which was maintained for the remainder of the experimental observation. Again, a preferential movement of water, in this instance out of the cornea, was suggested.

The magnitude of these temperature reversible changes was quite astounding. During incubation at 37°C. following refrigeration for 40 hours some 15 moles of corneal water per 100 gm. of dry weight were excreted in six hours. This is approximately 75 percent of the normal water content. If a 70 kg. man were to diurese at this rate from his extracellular fluid only he would excrete some two liters of urine/hour.

The temperature reversible studies demonstrated an active excretion of fluid from the cornea which could be subjected to measurement in the laboratory. The technique was hence applied to a study of various factors which modify the active process. In these studies isolated paired rabbit eyes were refrigerated under the imposed conditions at 0°C. After 40 to 45 hours one cornea was analyzed while the other eye was placed at 37°C. for an additional six hours before analysis. Results here presented are generally computed in terms of percent recovery, that is, that percent of sodium and water which entered the cornea at 0°C. which was subsequently excreted at 37°C.

### The relative importance of the endothelial and epithelial barriers to the maintenance of corneal hydration

There is general agreement from both clinical and experimental studies that damage to the endothelium causes a marked corneal hydration, whereas, epithelial damage is better tolerated. Of the two barriers the epithelium passes water and solutes much less rapidly. Recently Maurice and Giardini[20] demonstrated that removal of the epithelium in vivo was followed by an increase in corneal thickness to some twice the normal value. As the epithelium regenerated the thickness decreased but the central area remained maximally hydrated until at least the peripheral 50 percent of the surface epithelized. The results are subject to one or both of two significant interpretations; first, the epithelium acts simply as a barrier to the movement of lacrimal fluid or, second, the epithelium provides energy for the active dehydration. The temperature reversible system provided an excellent test of the possibilities. When the epithelium was completely removed there was a reduction in the capacity of the refrigerated cornea to excrete water and sodium into the aqueous during subsequent incubation at 37°C.[18] This, however, was not marked. Interestingly, when the central 50 percent of the epithelium was removed the excretion of water was not appreciably affected. The greatest influence on the dehydrating mechanism was exerted by the peripheral 50 percent. The reason for this is not clear. A proximity to limbal vessels may provide a functional necessity.

It is perhaps surprising that the epithelium has so little effect on the corneal excretory mechanism since this structure supplies the bulk of the energy of the total cornea. It cannot be stated from the present results that such epithelial influence as does exist results from an active transport of fluid across epithelial barriers. In any event the major contribution of the epithelium to the maintenance of normal corneal hydration must derive from the fact that it serves as a barrier against the lacrimal secretions.

On the other hand the endothelium acts not only as a barrier but appears to be the structure across which the active dehydration largely occurs. Two different experimental situations portray rather well the influence of this cellular layer. In the first a fraction of the endothelium was removed by introducing a needle at the limbus and scraping

TABLE 1

EFFECT OF LOCALIZED ENDOTHELIAL DAMAGE ON TEMPERATURE REVERSIBLE WATER AND CATION SHIFT OF THE RABBIT CORNEA

| Procedure | No. of Eyes | Water (moles/100 gm. dry weight) | | Sodium (mEq/100 gm. dry weight) | | Potassium (mEq/100 gm. dry weight) | |
|---|---|---|---|---|---|---|---|
| | | Control | Endothelial Damage | Control | Endothelial Damage | Control | Endothelial Damage |
| Fresh tissue | 93 | 20.0±1.2 (78.2%) | | 55.2±4.2 | | 10.5±1.3 | |
| 40 hr. at 0°C. | 12 | 35.7±2.7 (86.4%) | 43.3±8.5 (88.5%) | 80.8±6.4 | 95.8± 6.5 | 19.3±4.2 | 24.1±3.0 |
| 40 hr. at 0°C. plus 6 hr. at 37°C. | 12 | 19.1±3.0 (77.2%) | 28.0±7.9 (82.5%) | 55.9±3.2 | 73.5±15.3 | 15.3±2.5 | 18.0±3.3 |

the central few millimeters three strokes. In this instance, the water content increased to some 43 moles/100 gm. of dry tissue instead of the normally observed 35 moles. Logically, the break in the barrier permits a more ready access of the aqueous to the corneal stroma. During subsequent incubation at 37°C. the cornea thus damaged retained a marked ability to dehydrate (table 1). Although a return to normal hydration was not observed, the total excretion of fluid was of the same order of magnitude as that observed in the control eye in which the anterior chamber was penetrated without touching the endothelium.

A much greater hydration at 0°C. resulted when the posterior surface of the cornea was rubbed as completely as possible with the smooth curved surface of a 27-gauge needle (table 2). The results were quite variable, due, no doubt, to the difficulty in producing a standardized lesion. The hydration approached that measured when excised corneal pieces were immersed in fluid at the same temperature. During subsequent incubation of these endothelial damaged, but otherwise intact eyes, restoration towards normal hydration occurred. Indeed the total water excreted was again of the same magnitude as in the control eye which had been punctured at the limbus without disturbing the posterior surface. The results may reflect the fact that the endothelium was not completely removed. Indeed, our microscopic sections usually showed an adherent cellular layer. It is also possible, however, that the

TABLE 2

EFFECT OF GENERALIZED ENDOTHELIAL MASSAGE ON TEMPERATURE REVERSIBLE WATER AND CATION SHIFT OF THE RABBIT CORNEA

| Procedure | No. of Eyes | Water (moles/100 gm. dry weight) | | Sodium (mEq/100 gm. dry weight) | | Potassium (mEq/100 gm. dry weight) | |
|---|---|---|---|---|---|---|---|
| | | Control | Endothelial Massage | Control | Endothelial Massage | Control | Endothelial Massage |
| Fresh tissue | 93 | 20.0±1.2 (78.2%) | | 55.2±4.2 | | 10.5±1.3 | |
| 40 hr. at 0°C. | 12 | 35.7±2.7 (86.4%) | 60.6±7.6 (91.5%) | 80.8±6.4 | 139.5±19.1 | 19.3±4.2 | 24.1±2.7 |
| 40 hr. at 0°C. plus 6 hr. at 37°C. | 12 | 19.1±3.0 (77.2%) | 45.4±8.0 (88.8%) | 55.9±3.2 | 121.4±22.5 | 15.3±2.5 | 20.2±4.0 |

endothelium serves largely as a barrier to the movement of aqueous into the cornea and that the excretory activity lies at some closely adjacent and undamaged site—probably Descemet's membrane.

From these experiments, it is apparent that one "leak" in the dam is not sufficient to outstrip the effectiveness of the entire "pump." The experimental situation is undoubtedly analogous to that of a penetrating injury of the cornea or other limited endothelial disturbance where localized swelling occurs.

In addition one must credit the possibility that a massage of the endothelium which does not physically remove the membrane may nonetheless reduce the effectiveness of the mechanisms which control corneal hydration. This massage effect can be envisioned as similar to that of the lens where a few simple strokes result in a disturbance of the cation equilibrium with no apparent dehiscence in the lens capsule.[18] Corneal edema following cataract extraction is likely a related occurrence. The corneal edema following trauma with no other apparent damage may be similarly explained.

The influence of the endothelium and epithelium emphasizes the importance of a barrier to the dehydration activity. The reduction in hydration that attends the incubation at 37°C. of a previously refrigerated eye cannot be attributed to a temperature influence on the physical state of the stroma.

### The influence of the oxygen tension on the dehydration mechanism

That corneal hydration is increased in the absence of oxygen has been shown by a number of workers employing the isolated eye or tissue either immersed in solution or in the moist chamber.[15, 19, 21] In addition the experiments of Smelser and his associates[22] have indicated that depriving the living eye of atmospheric oxygen results in increased hydration. A very definite dependence of the dehydration mechanism upon the oxygen tension was demonstrated by the temperature reversible technique (fig. 1). The most active process was observed when the oxygen tension was 95 percent. Atmospheric tension was also effective as was an oxygen tension equivalent to that of alveolar air (13 percent). Moreover, at the oxygen tension found in aqueous humor (seven percent) there was still an appreciable and physiologically effective active transport of fluid from the cornea. When, however, the oxygen tension was reduced substantially to zero the "pump" mechanism was markedly altered. In this instance there was a slight movement of water but none of sodium.

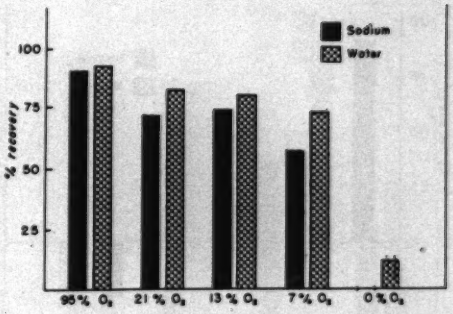


Fig. 1 (Harris). The effect of oxygen on the elimination at 37°C. of water and sodium from corneas of previously refrigerated rabbit eyes. The carbon dioxide concentration was five percent in the mixtures containing 95-percent $O_2$ and zero percent $O_2$ and six percent in all the others. Each bar represents the average result of at least eight determinations.

### Effect of metabolic poisons

Metabolic poisons promote corneal hydration. Philpot[23] injected a variety of such poisons, including cyanide, iodoacetate, fluoride and dinitrophenol into the anterior chamber of the isolated eye and incubated them at 32°C. in a moist chamber. Cyanide was only effective in fairly high (but uncertain) concentration and fluoride, iodoacetate and dinitrophenol at somewhat lower concentrations. Interestingly, she also found certain dyes to cause excessive hydration. Anticholinesterases were without significant effect. The interpretation of results obtained when enzyme poisons are injected into the intact

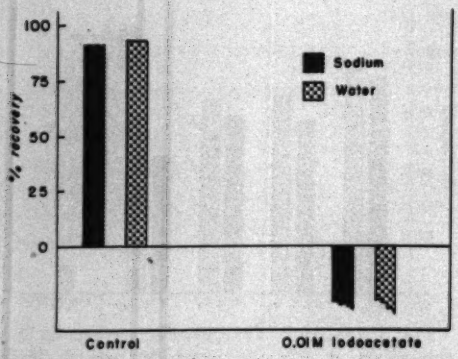


Fig. 2 (Harris). The effect of iodoacetate on the elimination at 37°C. of water and sodium from corneas of previously refrigerated rabbit eyes. The tubes were gassed with a mixture of 95-percent $O_2$ and five-percent $CO_2$. Each bar represents the average result of at least nine determinations.

eye is difficult for two reasons: first, the final effective concentration is not known and, second, involvement of other structures which might secondarily effect the corneal hydration must occur.

An example of the effect of metabolic poisons on temperature reversal studies is seen in Figure 2. In this instance 0.05 ml. of 0.01 M iodoacetic acid in a balanced salt solution was injected into the anterior chamber of one eye and a control solution into the other. The "pump" was blocked. The hydration which occurred during refrigeration was not particularly altered suggesting that there is no alteration in the permeability of the barrier at 0°C. (table 3). Subsequent to refrigeration and during incubation at 37°C. the cornea became further hydrated. Iodoacetate is a potent sulfhydryl poison. The importance of the sulfhydryl groups is also demonstrated by the observation that alloxan, also a sulfhydryl poison, will, when injected into the anterior chamber of the living rabbit, cause a marked corneal hydration.[24]

Rather parallel studies of the effect on corneal hydration when the isolated eye is immersed in solutions containing the poisons have given qualitatively the same results.[19] Where the metabolism is altered hydration is increased. In these instances as in the above cases the effective concentration delivered to the endothelial surface is unknown.

Using the temperature reversible technique three diuretics, one a mercurial and the other two nonmercurial* diuretics, have been studied. All are natriuretic. Chlorothiazide is a weak but probably physiologically insignificant carbonic anhydrase inhibitor.[25] The nonmercurial diuretics had little effect while the mercurial diuretic was quite potent (fig. 3). The results with the latter are particularly interesting in the light of some observations of Robinson[10] that the mercurial diuretics cause a hydration of tissue that cannot be attributed to migration of ions or

* Aminoisometradine was kindly furnished by Irwin C. Winter, M.D., G. D. Searle & Co. Chlorothiazide was kindly furnished by John R. Beem, M.D., Merck Sharp & Dohme Co.

TABLE 3

EFFECT OF IODOACETATE ON TEMPERATURE REVERSIBLE WATER AND SODIUM SHIFT OF THE RABBIT CORNEA

| Procedure | No. of Eyes | Content | | Concentration |
|---|---|---|---|---|
| | | Water (moles/100 gm. dry weight) | Sodium (mEq/100 gm. dry weight) | Sodium (mEq/1,000 gm. water) |
| 40 hr. at 0°C. | 9 | 34.5±1.7 (86.1%) | 79.0± 3.0 | 129.1±2.0 |
| 40 hr. at 0°C. plus 6 hr. at 37°C. | 10 | 40.3±2.7 (87.9%) | 105.2±11.7 | 148.1±3.6 |

block of active ion transport. His results, while not conclusive, suggest that the compound reduces water transport. Mercury is a sulfhydryl poison. It is possible that in our experiments an inhibition of oxygen was induced by the mercurial diuretic and the effect was therefore nonspecific. One should refrain, therefore, from interpreting these results as evidence for a water rather than a cation pump.

In general, studies with metabolic poisons have revealed little information not obtained from other studies. They indicate that respiration is essential to the active process and that glycolysis is also important. Information concerning any specific metabolic step which is involved in the dehydration process cannot be gleaned from the work. Nor can it be determined with any certainty from these studies which particular constituent is transported.

### The effect of the carbon dioxide tension and carbonic anhydrase inhibitors

During the course of our studies with oxygen tension it became apparent that the amount of carbon dioxide also influenced the ability of the cornea to dehydrate itself following a cold-induced hydration. The effect turned out to be more marked than we originally had anticipated (fig. 4). When carbon dioxide was excluded from the medium both the dehydration of the cornea and the movement of sodium from the cornea were at a maximum. With the carbon dioxide tension that would be anticipated in tissues (six percent), the movement of water and sodium was reduced. A still higher carbon dioxide level (10 percent) decreased the active process further. We have not employed higher concentrations but others have shown an atmosphere of carbon dioxide to be definitely toxic, more so than could be attributed to the anaerobiosis.[26]

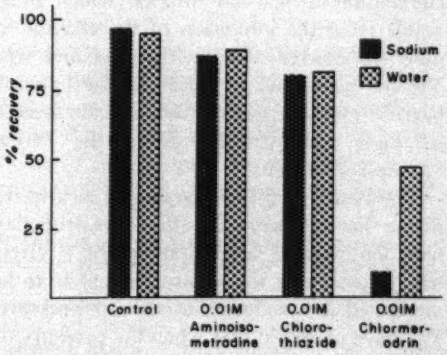


Fig. 3 (Harris). The effects of nonmercurial (aminoisometradine and chlorothiazide) and mercurial (chlormerodrin) diuretics on the elimination at 37°C. of water and sodium from corneas of previously refrigerated rabbit eyes. The tubes were gassed with a mixture of 95-percent $O_2$ and five-percent $CO_2$. Each bar represents the average result of at least eight determinations.

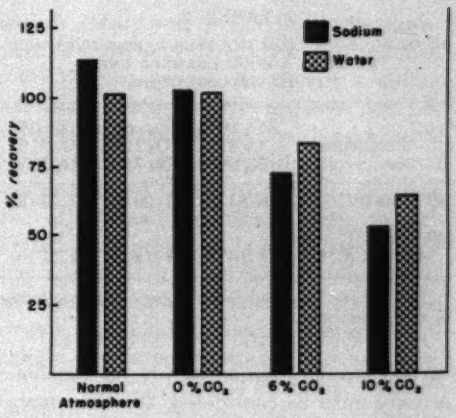


Fig. 4 (Harris). The effect of carbon-dioxide tension on the elimination at 37°C. of water and sodium from corneas of previously refrigerated rabbit eyes. All artificial mixtures contained 21-percent $O_2$ and the balance $N_2$. Each bar represents the average result of at least nine determinations.

The reason for the toxicity is not clear. As would be anticipated the pH of the aqueous was somewhat lower in those solutions of higher carbon dioxide tension (table 4). (The relatively lower pH at lower temperature is attributed to greater solubility of carbon dioxide in that temperature range.) A pH effect on collagen swelling, however, will not explain the difference since the hydration after refrigeration was substantially the same over the entire pH range. Other data is in agreement.[1] The effect of

TABLE 4

pH OF AQUEOUS FROM ISOLATED RABBIT EYES INCUBATED IN A MOIST CHAMBER UNDER INDICATED CONDITIONS

| Procedure | $CO_2$ 10% $O_2$ 21% $N_2$ 69% | $CO_2$ 6% $O_2$ 21% $N_2$ 73% | $CO_2$ 0% $O_2$ 21% $N_2$ 79% |
|---|---|---|---|
| 40 hr at 0°C. | 6.83 (7) | 7.00 (5) | 7.27 (8) |
| 40 hr at 0°C. plus 6 hr. at 37°C. | 7.10 (6) | 7.18 (5) | 7.37 (6) |

The figures in parentheses represent the number of determinations.

carbon dioxide tension or pH on metabolic activity must be considered. We have tentatively concluded, however, that the major influence of carbon dioxide is exerted by altering the hydrogen ion concentration at the particular barrier across which the transport of cation or water occurs without excluding the possibility of a direct toxic effect of carbon dioxide.

The deleterious effect both of an increased carbon dioxide tension and a reduced oxygen tension would feasibly be manifest when the lids are closed. On the other hand the increased temperature which must simultaneously occur would favor dehydration. Thus, in an isolated eye exposed to 95-percent $O_2$ and 5.0-percent $CO_2$ the dehydration mechanism was found to be more active at 37°C. than at 31°C., the supposed temperature of the exposed cornea.[16] The balance would appear to favor the temperature effect. Hydration, then, does not necessarily occur during long periods of lid closure.

The carbonic anhydrase inhibitors such as acetazolamide are of particular interest since the latter is often said to reduce corneal hydration in instances of such degenerative processes as endothelial dystrophy. We have accordingly utilized the temperature reversible shift in the study of these drugs. As in previous studies with enzyme poisons 0.05 ml. of 0.01 M concentration of the drug in appropriate solution were injected into the anterior chamber of one eye and a similar amount of balanced salt solu-

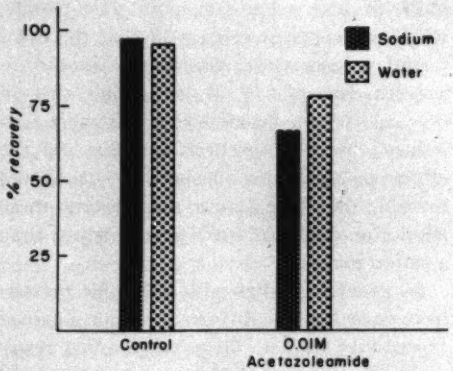


Fig. 5 (Harris). The effect of a carbonic anhydrase inhibitor on the elimination at 37°C. of water and sodium from corneas of previously refrigerated rabbit eyes. The tubes were gassed with a mixture of 95-percent $O_2$ and five-percent $CO_2$. Each bar represents the average result of at least nine determinations.

tion into the other. Acetazolamide* was found to reduce moderately both the migration of water and sodium from the cornea that had been hydrated during refrigeration at zero degrees (fig. 5).

Carbonic anhydrase is present in the cornea in only small concentration if at all.[27] The effect of the carbonic anhydrase inhibitors, therefore, likely is due to the accumulation of carbon dioxide which must result from the inhibition of the enzyme in other structures such as the lens and iris. Yet the possibility of action on the "pump" barrier must be considered. Acetazolamide in high concentration alters the cation "pump" of the lens.[13]

The results with acetazolamide clearly indicate that any beneficial effect this drug may have on corneal hydration is not a direct one. Most likely the clinical benefit is to be attributed to a reduced intraocular pressure. We have observed that when the pressure of the isolated eye is increased the ability of the cornea to recover from a cold-induced hydration is reduced.[17]

* Acetazolamide was kindly furnished by Gordon Dempsey, M.D., Lederle Laboratories Division, American Cyanamid Company.

### Which corneal constituent is actively transported?

An active dehydration having been definitely established it becomes of interest to determine, if possible, which corneal constituent is actively extruded,* an anion, a cation, or water. All three have been considered.

Of the anions probably only chloride requires mention. Changes in corneal hydration of the magnitude observed during incubation of the previously refrigerated eye are too great to be accounted for by an active transport of an organic ion or bicarbonate. Although we have not analyzed for it, chloride undoubtedly enters with sodium and potassium from the aqueous during refrigeration. During subsequent incubation at 37°C. if chloride were being actively transported a positive ion in equal amount would migrate so as to maintain electrical neutrality. Under this circumstance and, barring any mechanism of cation control, both sodium and potassium should reduce in amounts which depend on their relative concentrations in the stromal water. This was not observed. It seems unlikely, therefore, that chloride is the ion actively transported. The results also rule out the possibility of a primary potassium transport.

Of the remaining possibilities, the sodium "pump" and the water "pump," physiologic intuition would certainly favor the former. The readiness with which sodium leaves the previously refrigerated cornea during incubation at 37°C. would suggest a transport of sodium. Moreover, in all cases the movement of water and sodium are closely linked.

Of the two arguments against a sodium transport one is purely negative and the other, circumstantial. The first hinges on the fact that in no instance has the movement of sodium against an electrochemical gradient, that is, Donnan equilibrium, been demonstrated. This argument is not as strong as it may appear if it is acknowledged that (a) the Donnan ratio, stroma:aqueous, is not known, and (b) the stromal concentration of sodium can not be determined with accuracy.

The other argument is somewhat stronger and derives from the observation noted above that during incubation at 37°C. following refrigeration the sodium concentration rose sharply and reached a stable level, essentially that of fresh tissue, within two hours. Thereafter for the next four hours of our experimental situation the concentration remained constant in spite of the fact that the cornea was actively losing water and sodium and in spite of the fact that during this time the aqueous sodium concentration was rising. An active process involving the movement of sodium would seemingly require a control which would depend upon the concentration of sodium. Therefore, it is difficult to harmonize our observations with current concepts of factors controlling an active transport when sodium is considered to be the substance primarily moved. Put another way, what would make the dehydration process stop at normal hydration levels?

The remaining choice is an active transport of water. Adhering to our rigid definition, to accept this view one should demonstrate a movement of water against an osmotic gradient. This would require that the osmotic pressure of the corneal stroma exceed that of the aqueous. From his analyses Davson felt that such was the case.[30] We have tentatively arrived at the same conclusion. Making certain assumptions we estimated the osmotic strength of the stroma (exclusive of organic ions and nonelectrolytes) to be 327 mEq/1,000 gm. of water,[16]

* There is general agreement that movement of a substance against a chemical (or in the case of an ion, an electrochemical) potential is certainly an active transfer. This movement is "uphill"—water against an osmotic gradient, sodium against a gradient in excess of that predicted by the Donnan equilibrium, and so forth. However, the possibility that the metabolism may "assist" the movement of a substance in the direction of a gradient must be allowed. Such an assistance of glucose movement is seen in the lens[28] and other cells.[29] For the present, however, it would seem well to adhere to the first or more rigid definition since this definition envisages a system which is doing active work.

TABLE 5

SODIUM AND POTASSIUM CONCENTRATIONS OF EXCISED CORNEA IMMERSED IN A MODIFIED TYRODES SOLUTION AT 0°C. AND 37°C.

| Procedure | No. of Corneas | mEq/1000 gm. Water | |
|---|---|---|---|
| | | Sodium | Potassium |
| Fresh cornea | 93 | 154.0±7.0 | 29.3±3.8 |
| Immersed excised cornea refrigerated at 0°C. | | | |
| 10 hr. | 6 | 152.5±1.3 | 8.5±0.6 |
| 20 hr. | 6 | 154.7±1.0 | 8.6±0.6 |
| 40 hr. | 6 | 155.8±1.7 | 7.5±0.3 |
| 80 hr. | 6 | 155.7±3.3 | 7.6±0.3 |
| Incubated at 37°C. | | | |
| 6 hr. | 6 | 148.4±1.4 | 12.9±0.5 |
| 12 hr. | 6 | 158.2±1.7 | 10.7±0.3 |
| 18 hr. | 6 | 161.1±4.4 | 11.0±0.5 |

From analytical data Kinsey[31] estimated the osmotic activity of the aqueous to be equivalent to 155 mM/l. of sodium chloride or 310 mEq/l. as here calculated. His actual measurements of osmotic pressure were somewhat higher but still lower than the above calculated value.

Other evidence that the osmotic pressure of the cornea is somewhat higher than that of the aqueous was obtained when excised pieces of cornea were placed in a balanced salt solution containing a sodium concentration of 154 mEq/1,000 gm. of water at 37°C. Early in the course of incubation at 37°C. the sodium concentration of the cornea fell from a normal value of around 155 to about 148 mEq/1,000 gm. of water (table 5). The evidence then suggests that the cornea has a higher osmotic pressure than the aqueous.

The second piece of evidence which favors an active transport of water concerns the preferential movement of water into the cornea during refrigeration of the isolated eye and out of the cornea when the eye is subsequently incubated at 37°C. The apparent preferential movement of water into the cornea at 0°C. can be considered as due to movement along an osmotic gradient. The changes which occur subsequently at 37°C. are construed as indicative of a water "pump" re-establishing an osmotic gradient. Two other interpretations of these temperature effects which do not carry an active transport connotation are possible, however. These were considered at the time of our original publication but deserve emphasis here in greater detail.

The first concerns the possible increase in osmotic activity which occurs at low temperature and is best exemplified in the erythrocyte. Erythrocytes can be placed in a mildly hypotonic solution of such a concentration that no hemolysis will occur at 37°C., while complete hemolysis results when the mixture is chilled to 0°C. Moreover a reversible increase in erythrocyte volume can be demonstrated when the temperature is lowered, due presumably to an increase in osmotic activity at lower temperatures.[32] These changes cannot be attributed to cation shift. The phenomenon is similar to that seen in the cornea.

The erythrocyte change has been attributed to the fact that the disassociation of protein is reduced at the lower temperature, the cation so released forming the bicarbonate salt with a resultant total increase in osmotic activity. At higher temperatures the sodium salt of the protein is formed and the bicarbonate ions become carbonic acid and are lost as carbon dioxide. Carbonic anhydrase would catalyze the latter reaction. The increase in osmotic activity requires that there be no movement of the osmotically active material from the cell (or cornea).

Acceptance of this process in the cornea would hinge on demonstrating a higher cornea:aqueous cation ratio at 37°C. than at 0°C. Excised bits of cornea were incubated at 0°C. and at 37°C. in a balanced salt solution containing sodium in the amount of 153 and 154 mEq/1,000 gm. of water. The increase in hydration of the corneal pieces immersed at 0°C. was much greater than that observed when the intact eye was refrigerated in a moist chamber at 0°C. (fig. 6). The sodium concentration of the excised pieces became fairly constant at around 20 hours, thereafter maintaining a

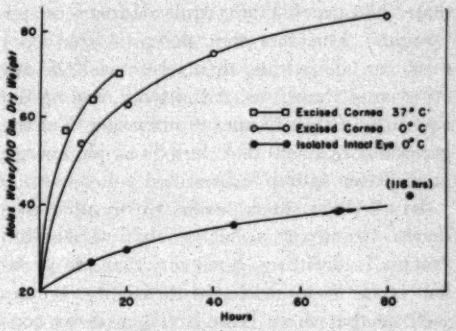


Fig. 6 (Harris). Hydration of the rabbit cornea under various conditions. The excised pieces of cornea (nine mm. in diameter) were immersed in balanced salt solution and gassed with a mixture of 21-percent $O_2$ and six-percent $CO_2$, the balance being made up of $N_2$. The isolated intact eyes were gassed with a mixture of 95-percent $O_2$ and five-percent $CO_2$ and refrigerated in moist chambers. Each point represents the average result of at least six determinations.

level of approximately 156 mEq/1,000 gm. of water (table 5). At 37°C. the sodium concentration did not reach constancy during the 18-hour period of observation. (More prolonged observation at 37°C. was deemed meaningless since the generation of acidic ions by metabolic processes would tend to obscure the true cation ratio.) However, the hydration observed after approximately 18 hours at 37°C. corresponded to that after 30 hours refrigeration, when a stable sodium level had been reached. Comparing then the corresponding sodium values, the corneal: media ratios were 161/154 at 37°C. as compared to 156/153 at 0°C. Because of the dilution of the corneal section this is not the in vivo Donnan ratio. (Stromal volume is so great in these pieces that in this instance the stromal values probably closely approximate the measured corneal values.)

The dilution of the cations on refrigeration of the intact eye that can be attributed to movement of water amounted to 10 mEq/1,000 gm. of water. The protein concentration in these corneas, however, is somewhat less than half that of the excised bits, due, of course, to greater swelling of the latter. Consequently, while admitting that the sodium concentration of the excised bits incubated at 37°C. may be erroneously high because of metabolic activity, one can only accept with reservations the view that the preferential movement of water with temperature change results from a water "pump."*

The second nonactive process which must be considered in temperature reversal studies involves the question of bound water, that is, nonsolvent water. If bound water is present in the cornea we are measuring it as well as the solvent water by our technique of drying the cornea at 105°C. to constant weight. The amount of water bound by any protein is, of course, greater at the lower temperatures. During the first 20 hours of refrigeration as we have mentioned some 20 percent of the movement of fluid is water. If this were due to an increase in bound water it would mean a "binding" of two moles of water/100 gm. of dry tissue. The net effect would be a reduction in solvent water and an increase in osmotic activity. The whole subject of bound water in living tissues or even in protein solution is highly controversial since its measurement is difficult.[33] There is no proof that such bound or nonsolvent water is normally present in the cornea. If it were, the osmotic activity of the cornea would be greater than now calculated since it is unlikely that there are bound, that is osmotically inactive inorganic cations or anions. From lack of evidence therefore, the role of bound water in these temperature changes must remain in doubt.

* There is one other observation which bears on this question. The proposed effect would seemingly require an active carbonic anhydrase if the change in osmotic activity with the temperature is to occur at a constant pH. The cornea contains at best small amounts of carbonic anhydrase, yet in the presence of carbonic anhydrase inhibitors the sodium diluted to the anticipated *concentration* in the cold, namely, to around 136 mEq/1,000 gm. of water, and when replaced at 37°C. again concentrated to approximately 155 mEq/1,000 gm. of water even though the over-all movement of sodium and water was somewhat decreased. This result would suggest that the preferential movement of water is not due solely to a shift in the Donnan ratio.

An active transport of water cannot be accepted unless an adequate energy source can be demonstrated. Since the turnover of water across the corneal surfaces is undoubtedly rapid as compared to ions, a water "pump" has been considered to be energetically wasteful as compared, for example, to a sodium "pump." Actually this is not the case. Maurice[6] calculated the energy requirement for a sodium "pump," accepting a small concentration gradient from cornea to aqueous and using his figures for $Na^{24}$ exchange. He found the needed energy to be only a small fraction of that available. Assuming an osmotic gradient of a similar degree (but in the reverse direction) it can be calculated that the same expenditure of energy would support a water "pump" if the turnover of water was 160 times as rapid as sodium. This is probably a generous margin since it is very likely that the relative rate of turnover of water and sodium is less than this figure. A water "pump" is then not more extravagant than a sodium "pump."

Similarly the energy required for the excretion of water during incubation at 37°C. of the previously refrigerated eye can be calculated (employing the equation $w = nRT\ln N_2/N_1$, where n is the number of moles of water moved/unit time and $N_2$ and $N_1$ are respectively the mol fractions of water in aqueous and cornea). Assuming the osmotic pressure of the cornea to be 15 milliosmoles higher than the aqueous, the energy required to excrete 15 moles of water/100 gm. dry weight in six hours is $5.2 \times 10^{-3}$ cal./min./100 gm. dry weight. The energy available calculated from the data of Robbie, Leinfelder, and Duane[34] is 3.8 cal./min./100 gm. dry weight. The figures from the data of Langham[35] are somewhat higher, that is, 7.2 cal./min./100 gm. of dry weight. In any event only a fraction of the total available energy is needed. When the epithelium is removed the oxygen consumption falls to approximately one third according to the data of the former authors. Thus even without the epithelium the energy available is more than 200 times that required for a water "pump." The fact that de-epithelized corneas can dehydrate themselves well is not surprising therefore. Admittedly, use of the equation in this manner is not exact. Yet the general conclusion that there is ample energy for a water "pump" is justified.

In all, then, there seems to be more evidence to favor a water than a sodium "pump," admitting, however, that the problem is far from settled with finality. It may well be that there is an active process controlling both water and sodium which, as mentioned above, some postulate for the cell and which may occur in mitochondria.[36]

According to our present concept the cornea tends to swell because of the Donnan osmotic pressure, the cornea acting much like a large single cell whose membrane is permeable to all major readily diffusible ions and nonelectrolytes. Swelling is prevented by the excretion of water from the cornea, an osmotic pressure differential thereby being created with a gradient from cornea to aqueous. Sodium and other ions will diffuse along the concentration gradient from the stroma to the aqueous. The active process occurs for the most part at the endothelial surface, the epithelium playing at most a minor role. The magnitude of the corneal dehydration process is great indeed. The "pump" can certainly account for diffusion via the limbal vessels. Moreover, the activity is probably sufficient to prevent generalized swelling from localized trauma to the endothelium. In this instance the rate of diffusion of water and salt through the stroma is balanced by excretion across the normal endothelium.

## The effect of hyaluronic acid sulfate on corneal hydration

There is one other facet to this problem which has received some attention but is little understood. This concerns the relationship of the polysaccharide of the cornea to corneal swelling. It has been shown by others that the swelling of collagen tissues, including the cornea, is considerably reduced at

physiologic pH when the polysaccharide is removed.[1] It seems reasonable to suppose that relationship between corneal hydration and the polysaccharide must carry over to the living state and that in particular the interaction of polysaccharide and collagen will determine to a certain degree the hydration of the cornea as well as its transparency.

We have recently been working with a polysaccharide which came to our attention because of its antihyaluronidase activity. This compound is the sulfate of hyaluronic acid prepared by sulfating a fairly purified hyaluronic acid under conditions that are not too rigorous. The final product has an elemental analysis which indicates that there are three sulfates present on each disaccharide unit. This differs, therefore, from the usual polysaccharide sulfate such as heparin which has only two sulfates per repeating unit. The compound has definite antihyaluronidase activity as measured by the viscosity reducing technique. As would be expected, too, it has anticoagulant properties and in certain other respects is similar to the sulfated polysaccharides such as heparin.*

In the following experiments we have employed a concentration of 100 mg./ml. In such concentration the material has a sodium value of approximately 500 mEq/l. but has an osmotic pressure somewhat less than 200 milliosmoles/l.—approximately two thirds that of aqueous humor. This low osmotic pressure attests to the fact that the compound is still highly polymerized.

Hyaluronic acid sulfate causes a fairly profound corneal hydration following intravitreal injection in vivo (fig. 7). When 0.05 cc. of the above solution was injected into the vitreous of the living rabbit on each of two successive days the eye generally remained quiet for several days. Hydration of the cornea first appeared around seven days after the second injection. The rapidity with which the hydration was seen varied to a certain extent. Hydration could be hastened by giving three intravitreal injections on successive days. In these cases corneal hydration was observed within three days after the last injection. The swelling was not necessarily uniform over the cornea and was generally maximum around ten to fourteen days. It then began to subside. The hydration was more pronounced in those eyes which had received three injections than in those receiving only two (table 6). The degree of induced hydration was quite variable, in some cases being barely appreciable.

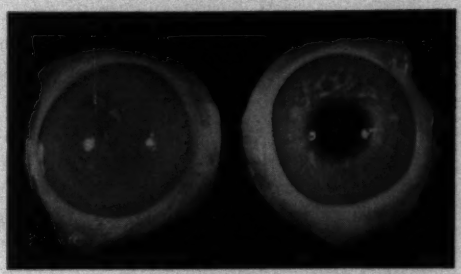

Fig. 7 (Harris). Corneal hydration following intravitreal injection of hyaluronic acid sulfate. The eye on the left had received two injections on successive days of 5.0 mg. of hyaluronic acid sulfate in 0.05 ml. The eye on the right had received 5.0 mg. of heparin in 0.05 ml. Eyes were enucleated 17 days after second injection.

TABLE 6

IN VIVO HYDRATION OF CORNEA FOLLOWING INTRAVITREAL INJECTION OF HYALURONIC ACID SULFATE INTO RABBIT EYE

| Procedure | Rabbit No. | Days after Last Injection | Water Content —% of Normal | |
|---|---|---|---|---|
| | | | O.S. | O.D. |
| Two injections on successive days | 539 | 8 | — | 123 |
| | 536 | 14 | 129 | 300 |
| | 537 | 14 | 132 | 141 |
| | 540 | 28 | 114 | 121 |
| | 541 | 28 | 124 | 97 |
| | 370 | 31 | — | 121 |
| | 443 | 95 | — | 77 |
| Three injections on successive days | 550 | 10 | 286 | — |
| | 551 | 10 | 370 | 192 |
| | 552 | 10 | 403 | 372 |

* The hyaluronic acid sulfate and the data concerning it were kindly furnished by Robert L. Craig, M.D., G. D. Searle and Company.

ble and in others quite marked. The variability probably reflects the fact that the intraocular distribution of injected material was not uniform. The maximum hydration we observed following intravitreal injection amounted to an increase in water to some 400 percent of the normal value. This increase is about three times that seen when the isolated eye is refrigerated in a moist chamber.

The hydration caused by the compound was even greater when the substance was injected into the anterior chamber (fig. 8). In this instance 0.05 cc. of the solution was injected into the anterior chamber with a 30-gauge needle flushing back and forth two or three times while the needle was in place. The increased hydration in these instances was generally observed within 24 to 48 hours after the second injection and reached a maximum at an earlier period than when injection was made into the vitreous. Controls receiving saline injections showed no such change. Moreover, injection of the same quantity of heparin (100 mg./ml.) into the anterior chamber caused little or no alteration in the hydration of the cornea (table 7).

When injection was made into the cornea using a 30-gauge needle so placed as to inject 0.05 cc. of the material approximately into midstroma, hydration was fairly marked within 24 hours after the injection. No such hydration, incidentally, was observed if the control eye was injected with normal saline. When the control eye was injected with an equal amount of heparin some hydration of the cornea was observed. This was most marked within the first few days after injection but subsided fairly rapidly, whereas, the cornea injected with the hyaluronic acid sulfate remained fairly hydrated (table 7; fig. 9).

TABLE 7

IN VIVO HYDRATION OF CORNEA FOLLOWING INJECTION OF HYALURONIC ACID SULFATE OR HEPARIN INTO INDICATED STRUCTURE OF RABBIT EYE

| Procedure | Rabbit No. | Days after Injection | Water Content —% of Normal | |
|---|---|---|---|---|
| | | | O.S.: Heparin | O.D.: Hyaluronic Acid Sulfate |
| Anterior chamber: two injections | 389 | 5 | 86 | 209 |
| | 390 | 5 | — | 529 |
| | 393 | 5 | 113 | 347 |
| | 394 | 5 | 105 | 581 |
| | 395 | 5 | 87 | 522 |
| Intracorneal: one injection | 577 | 7 | 130 | 242 |
| | 578 | 7 | 94 | 162 |
| | 586 | 7 | 93 | 193 |

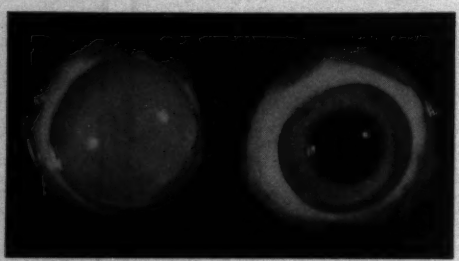

Fig. 8 (Harris). Corneal hydration following injection of hyaluronic acid sulfate into the anterior chamber. The eye on the left had received two injections on successive days of 5.0 mg. of hyaluronic acid sulfate in 0.05 ml. The eye on the right had received 5.0 mg. of heparin in 0.05 ml. Eyes enucleated five days after second injection.

The above results have been obtained only in vivo and as can be seen the changes were fairly slow to develop. When 0.05 cc. of this material was injected into the anterior chamber of the isolated eye and the eye subjected to the temperature reversible studies previously described, quite a different picture was obtained. Under this circumstance the cornea hydrated to only half the value usually observed during refrigeration (table 8). In other words this substance in some way prevented the entrance of fluid from the anterior chamber into the cornea. During subsequent incubation of these eyes at 37°C. an appreciable excretion of fluid from the cornea was seen, although the corneal water did not quite approach the value of the control eye which had been in-

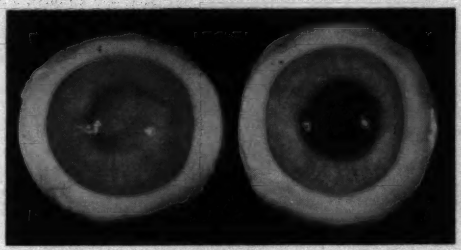

Fig. 9 (Harris). Corneal hydration following intracorneal injection of hyaluronic acid sulfate. The eye on the left had received one injection of 5.0 mg. of hyaluronic acid sulfate in 0.05 ml. The eye on the right had received 5.0 mg. of heparin in 0.05 ml. Eyes enucleated 17 days after injection.

jected with 0.05 cc. of a balanced salt solution.

On the other hand the compound did not appreciably alter the degree of hydration of excised bits of cornea immersed in solution at 37°C. (table 9). Thus after 18 hours in a mixture contaning 15 cc. of balanced salt solution to which had been added 0.25 cc. of the hyaluronic acid sulfate, the degree of hydration was substantially that of the control. The sodium concentration of the cornea was somewhat higher than that measured in the control. This does not represent any difference in the cation ratio since the sodium concentration of the hyaluronic acid sulfate solution was generally some 5 mEq/1,000 gm. of water higher than that of the parent salt solution.

The differing actions of this compound, in vivo as compared to in vitro, present some interesting possibilities.

In the acute experiment in the isolated eye the compound is seen to reduce the movement of fluid into the cornea. (In all of our experimental studies, involving over a thousand eyes, this is the only situation in which the cold-induced hydration has been observed to be significantly reduced.) In the chronic experiment in vivo injection into the anterior chamber is followed in a few days by a marked accumulation of fluid in the cornea. We have tended to rule out the possibility that the observed effects of this drug are due to its antihyaluronidase activity. In vivo, for example, the changes come on gradually and are not characteristic of enzyme inhibition.

An alternative explanation is that by virtue of its great anionic strength hyaluronic acid sulfate replaces some of the polysaccharide of the cornea or at least of the endothelial-Descemet's barrier. Because of the size of the molecule, penetration into the cornea would seem unlikely. The possibility that it may simply attach to collagen as additional polysaccharide must also be considered. In the acute in vitro experiment the reduction in rate of fluid movement into the cornea would be compatible with this concept.

TABLE 8

EFFECT OF HYALURONIC ACID SULFATE ON TEMPERATURE REVERSIBLE WATER AND CATION SHIFT OF THE RABBIT CORNEA

| Procedure | No. of Eyes | Water (moles/100 gm. dry weight) | | Sodium (mEq/100 gm. dry weight) | | Potassium (mEq/100 gm. dry weight) | |
|---|---|---|---|---|---|---|---|
| | | Control | Hyaluronic Acid Sulfate | Control | Hyaluronic Acid Sulfate | Control | Hyaluronic Acid Sulfate |
| Fresh tissue | 93 | 20.0±1.2 (78.2%) | | 55.2±4.2 | | 10.5±1.3 | |
| 40 hr. at 0°C. | 10 | 34.6±2.7 (86.1%) | 27.5±1.9 (83.1%) | 82.1±9.4 | 71.7±5.2 | 17.8±3.3 | 13.0±1.3 |
| 40 hr. at 0°C. plus 6 hr. at 37°C. | 9 | 20.7±3.1 (78.6%) | 21.7±2.2 (79.4%) | 56.1±7.0 | 64.1±8.9 | 14.7±1.5 | 11.7±1.0 |

TABLE 9

EFFECT OF HYALURONIC ACID SULFATE ON WATER CONTENT AND SODIUM AND POTASSIUM CONCENTRATIONS OF EXCISED CORNEA INCUBATED FOR 18 HOURS AT 37°C.

| Procedure | No. of Eyes | Moles/100 gm. Dry Weight | mEq/1000 gm. Water | |
|---|---|---|---|---|
| | | Water | Sodium | Potassium |
| Fresh cornea | 93 | 20.0±1.2 (78.2%) | 154.0±7.0 | 29.3±3.8 |
| Excised cornea immersed in modified Tyrode's ($Na^+$=153 mEq/liter) | 6 | 69.9±1.0 (92.7%) | 161.1±4.4 | 11.0±0.5 |
| Modified Tyrode's Plus Hyaluronic Acid Sulfate ($Na^+$ = 160 mEq/liter) | 6 | 71.3±5.2 (92.8%) | 166.1±3.4 | 10.8±0.4 |

In the more chronic in vivo study, however, the cornea ultimately becomes hydrated. This could result either from an increase in barrier permeability or a decrease in "pump" activity. It is to be remembered that Descemet's membrane contains a polysaccharide. It is likely that this polysaccharide barrier is intimately involved with the "pump" process just as the lens capsule is involved with the cation "pump" in the lens. (It is germane to our thesis to mention that cataracts eventually develop in the eyes in which the substance has been injected into the vitreous. Here again some exchange or addition to the polysaccharide of the capsule seems reasonable.) Whatever the explanation the above results indicate that the polysaccharide-collagen system is intimately connected with corneal hydration in vivo as well as in vitro.

Fig. 10 (Harris). Anterior stromal opacity following intravitreal injection of hyaluronic acid sulfate. The eye on the left had received two injections on successive days of 5.0 mg. of hyaluronic acid sulfate in 0.05 ml. The eye on the right had received 5.0 mg. of heparin in 0.05 ml. Eyes enucleated 17 days after second injection.

The compound produces a variety of other effects in the eye over a period of time. Among them is another corneal change which I would like to touch on just briefly. Following injection either into the vitreous or the anterior chamber most of the eyes ultimately developed an opacity in the anterior stroma (fig. 10). This opacity, as one viewed it under the slitlamp, lay just underneath the epithelium and generally was centrally located although on occasion was peripheral. It was not particularly related to the hydration and was seen in corneas which had hydrated minimally. These anterior stromal opacities first appeared some seven to 10 days after the second intravitreal injection, although sometimes their onset was more delayed. They did not occur in every eye. Generally speaking these anterior stromal opacities tended to reduce over a period of several weeks. Similar but less extensive changes were observed following the injection of heparin.

On histologic examination four changes were observed although all were not necessarily present in the same eye. First, the regular laminated pattern of the collagen fibers in the anterior stroma was lost. Second, there was an increase in nuclear material suggesting keratoblastic activity. Usu-

ally, no inflammatory cells were seen. Third, immediately under the epithelium a clear amorphous material with the histologic characteristics of a Bowman's membrane occasionally developed. (Bowman's membrane is not normally present in the rabbit eye.) Fourth, the epithelium became uneven and considerably reduced in thickness in certain instances.

It is rather difficult to explain why these opacities develop anteriorly and why they develop most readily in the central portion of the cornea. It would seem unlikely that the total molecule has diffused across the endothelial surface and into the cornea. The final mode of action must await studies with isotopically labeled material and other techniques.

## Summary

This review of the current status of our knowledge concerning corneal hydration has emphasized the following:

1. The hydration of the cornea is under metabolic control. The metabolic process requires oxygen. The oxygen supply normally available from the aqueous humor is sufficient to maintain a fairly effective "pump" mechanism and it is only when the oxygen is completely depleted that a serious handicap in the mechanism results.

2. The endothelial, rather than the epithelial, surface is the major site of the active process.

3. Potent metabolic poisons cause a marked decrease in the "pump" mechanism and a hydration. Mercurial diuretics also inhibit the "pump" activity, a fact which may be related to nonspecific inhibition of respiration rather than specific inhibition of a "pump" mechanism.

4. The system is carbon dioxide sensitive, an increase in carbon dioxide tension reducing the active transport. Carbonic anhydrase inhibitors reduce the "pump" activity to a slight degree, perhaps by reason of the same mechanism.

5. The arguments in favor of a water rather than a sodium "pump" have been weighed. Analysis of current data indicates that water is the particular corneal constituent which is actively excreted from the cornea. The possibility of a sodium "pump" has not been excluded. The energy requirements for a water "pump" can easily be met by the cornea, even if the epithelium is removed.

6. The hydration properties of the cornea can be altered by local injection of an anionic polysaccharide. This may occur by altering Descemet's membrane or the Descemet's-endothelial complex with change in permeability or decrease in active transport.

*3181 SW Sam Jackson Park Road (1).*

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# ON THE ENZYMOLOGY OF THE REFRACTORY MEDIA OF THE EYE*

## V. On the occurrence of proteolytic enzymes in normal and cataractous lenses[1]

E. Albert Zeller, M.D., and Anima Devi, Ph.D.
*Chicago, Illinois*

The lenticular metabolism not only has to build up and maintain the high protein level of the lens—the highest of all tissues—but also to keep the proteins in such a chemical and physical state that transparency is secured. Since a great variety of situations, namely, ionizing radiation, galactosemia, and nutritional deficiencies, lead to cataract formation, it is concluded that the chemical composition and physical state of the lens proteins are the result of a dynamic equilibrium. Any serious disturbance of the systems responsible for this equilibrium must, therefore, change the composition or physicochemical state of the lenticular proteins. Under these circumstances the latter will revert to a more probable state, as manifested by the loss of the transparency.

While there is no unambiguous evidence available, that the proteinases (endopeptidases) and peptidases (exopeptidases) are essential components of the systems which keep the cell proteins in their dynamic equilibrium, a large body of information is in harmony with this concept. Thus new information on the protein metabolism of the lens may be obtained by studying the lenticular peptidases under physiologic and pathologic conditions.

A few determinations of some exopeptidases of the lens were made previously[2,3] so that established methods could be applied for the present problem. However, no direct observations about the occurrence of endopeptidasis were found in the literature.

Following the autolysis of beef lens homogenates over a period of 79 days, Krause[4] noticed the decrease of the protein nitrogen. These results suggested the presence of proteinases in the lens. By using sensitive methods we were able to show definitely that enzymes of this type do occur in the lens. Thus the field of a detailed analysis of the lenticular endopeptidases was opened.

### Manometric determination of exopeptidases

With this method one enzyme—the L-peptidase—is measured with the help of a second one—ophio-L-amino acid oxidase.[5] L-peptides are hydrolyzed under the influence of exopeptidases (lens homogenate). As soon as certain free amino acids appear in solution, they are oxidized by the powerful L-amino acid oxidase found in snake venoms.[6,7] When the components are properly chosen, the rate of oxygen uptake and ammonia formation are proportional to the peptidase activity. The former is determined conventionally with the Warburg apparatus, the latter with the help of Conway units. The system of measurement is given in Table 1.

The reaction rates are given in form of Q-values and are expressed in terms of microatoms oxygen used or micromoles of ammonia liberated per hour and per gram fresh tissue.

### Manometric determination of endopeptidases

Certain amino-acid esters turned out to be excellent substrates of proteinases, for example, L-phenylalanine ethylester (PAEE) is rapidly attacked by crystalline chymotrypsin. When the hydrolysis of this ester takes place in a bicarbonate medium, one mole

* From the Department of Biochemistry, Northwestern University Medical School. This study was supported in part by funds provided under contract AT (11-1) 118 between Northwestern University and the U.S. Atomic Energy Commission.

TABLE 1

COMPOSITION OF THE SYSTEM FOR THE MEASUREMENT OF LENS EXOPEPTIDASES

| |
|---|
| 0.3 ml. lens homogenate (1:6, saline) |
| 0.2 ml. venom solution (0.4 mg. venom of Vipera russelli, saline) |
| 0.1 ml. of 0.01 M $MnCl_2$ (saline) |
| 0.4 ml. of 0.01 M substrate (0.01 M phosphate buffer pH 7.2) or buffer alone |

of $CO_2$ is liberated for each mole of hydrolyzed ester. In this way chymotrypsin[8] and intracellular proteinases with the same substrate pattern as chymotrypsin (chymotrypsinases, cathepsins c) can be measured manometrically. The conditions of measurement are summarized in Table 2.

The reaction velocity is expressed in terms of micromoles of $CO_2$ liberated per hour and per gm. fresh tissue. So-called substrate blanks as well as enzyme blanks are carried along with the complete systems as indicated in Table 2.

A similar method for the measurement of trypsin and trypsinases using benzoyl-L-arginine ethylester (BAEE) as a substrate, was developed in this laboratory.[9]

## MATERIALS

Homogenates of cataractous and normal human lenses were prepared from surgically removed lenses or from eyes obtained from an eye-bank.* In transit to the laboratory the lenses were kept in saline. When the lenses or eyes were not used immediately for experimentation, they were stored at −20°C. The preparation of lens homogenates has been described in a previous paper.[10]

One eye of each rabbit was irradiated with 2000 r, while the rest of the animal was protected against the X rays. The other eye served as a control. The eyes were removed several months after the irradiation, so that fully matured cataracts developed.

The peptides were purchased from Hoffmann-LaRoche Inc., Basel, and the amino-acid esters were synthesized in this laboratory.

TABLE 2

COMPOSITION OF THE SYSTEM FOR THE MANOMETRIC MEASUREMENT OF CHYMOTRYPSINASES

| |
|---|
| 1.0 ml. lens homogenate (1:3, Ringer-solution) or Ringer-solution alone |
| 0.7 ml. $NaHCO_3$ (0.18 M) |
| 0.3 ml. distilled water |
| 1.0 ml. 0.075 M PAEE (in Ringer-solution) or Ringer-solution alone |

TABLE 3

L-PEPTIDASES IN HUMAN LENS

| Substrate | Number of Determinations | $Q_{O_2}$ | $Q_{NH_3}$* |
|---|---|---|---|
| GL | 4 | 9.7 | 3.4 |
| LG | 4 | 3.5 | 1.9 |
| LGG | 2 | 2.0 | 0.8 |

* Production of ammonia measured after one hour of incubation.

## HUMAN LENSES

As previously demonstrated for rabbit eyes,[3] the human lens is capable of catalyzing the hydrolysis of the three peptides glycyl-L-leucine (GL), L-leucyl-glycin (LG) and L-leucylglycylglycin (LGG). In both species glycyl-L-leucine is attacked more rapidly than the two other peptides (table 3).

No reduction of peptidase activity is found in cataractous lenses (table 4).

An enzyme attacking PAEE occurs in the human lens. The rate of hydrolysis is

TABLE 4

L-PEPTIDASES IN CATARACTOUS HUMAN LENS

| Substrate | Number of Determinations | $Q_{O_2}$ | $Q_{NH_3}$* |
|---|---|---|---|
| GL | 6 | 14.6 | 3.8 |
| LG | 6 | 5.3 | 2.8 |

* Production of ammonia measured after one hour of incubation.

* We are indebted to the Eye-Bank of the Illinois Society for the Prevention of Blindness and to Dr. David Shoch, Department of Ophthalmology, Northwestern University, for providing us with the valuable human material.

TABLE 5

DEGRADATION OF PAEE BY LENS

| | Number of Determinations | $Q_{CO_2}$ |
|---|---|---|
| Man, normal | 10 | 37 |
| Man, cataract | 11 | 13 |
| Cattle, normal | 5 | 41 |
| Rabbit, normal | 17 | 137 ± 80 |
| Rabbit cataract (2000 r) | 17 | 30 ± 40 |

TABLE 6

PEPTIDASE ACTIVITY IN RABBIT LENS

| Lens | Substrate | Number of Determinations | $Q_{O_2}$ | $Q_{NH_3}$ |
|---|---|---|---|---|
| Normal | GL | 13 | 16.2 | 4.2 |
| Normal | LG | 11 | 6.2 | 3.1 |
| Cataract | GL | 4 | 1.8 | 2.0 |
| Cataract | LG | 3 | 1.4 | 0.3 |

of the same order as found in other species (table 5). In cataractous lenses a marked drop of the proteinase activity as compared with normal lenses is observed.

## RABBIT LENS

In the lens of rabbits which are rendered cataractous by X rays, the peptidase activity is much lower than that present in normal lenses (table 6). The same is true for the chymotrypsinase activity (table 5).

Notwithstanding the considerable variations of the activity within a group of apparently normal lenses, the difference between normal and pathologic lenses is significant.

## CONCLUSIONS

By this study several gaps of the enzyme catalogue of the lens were filled out. For the first time the existence of a lenticular proteinase (cathepsin c, chymotrypsinase) was proven by direct measurement.* The activity of this enzyme is lower in cataractous as compared with normal lenses. Whether this change of enzymic activity precedes or follows the formation of the cataract is not known as yet.

It was previously shown that in rabbit lens various peptidases attack the peptides glycyl-L-leucine (GL), L-leucylglycin (LG) and L-leucylglycylglycin. Similar enzymes seem to occur in the human lens. While the enzyme activity of the normal human lens is the same as in the cataractous material, the peptidase activity of the rabbit lens rendered cataractous by X rays is reduced. It is not known whether this difference is due to the type of cataract—spontaneous vs. X ray-produced—or due to the kind of species.

*303 East Chicago Avenue (11).*

* Only very small activity of a trypsinase was observed in cattle lens.

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## DISCUSSION

DR. JIN H. KINOSHITA (Boston): One of the most striking things about this paper is the high activity of the protease observed in the lens. This finding is in conflict with the study by Krause who found that proteases are not particularly active in the lens.

I wonder how specific this method is for chymotrypsin. The substrate used for this enzyme was phenylalanine ester. Since various peptidases have been demonstrated to occur in lens exhibiting high enzymatic activities, is it possible that rather than the measurement of protease activity you are in fact measuring peptidase activity? Does not the substrate phenylalanine ester appear to be a more suitable substrate for peptidase rather than an enzyme which will split an entire protein molecule?

DR. E. ALBERT ZELLER (closing): In various laboratories, extensive studies have been made on the specificity of these enzymes, using crystalline trypsin, chymotrypsin, and so on. These amino-acid esters are excellent substrates of proteinases. They are such good substrates that a new specificity pattern could be established.

In contrast with textbook notions, we found that trypsin attacks only side chains which have arginine or lycine in them. It could also attack histidine. However, at the level at which these enzymes are studied, the predominant activity that we find on the order of μg. is only visible for these proteinases and not for peptidases. It might be that peptidases may have a slight action on these amino acidases, but they don't compare at all with the action of the proteinases; so, I think that in the present status of the field of knowledge of specificity pattern of this enzyme, there is no doubt that it is a cathepsin proteinase which is responsible for the hydrolysis of the phenylalanine ester.

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# THE TRANSCORNEAL POTENTIAL*

ALBERT M. POTTS, M.D., AND ROBERT W. MODRELL, M.S.
*Cleveland, Ohio*

The inverse relationship between corneal hydration and transparency has been accepted since its enunciation by Leber in 1873.[1] The mechanism for maintaining the dehydration necessary for transparency has been and remains a subject for speculation.[2-4] The dependence of the dehydration mechanism on metabolic activity has been demonstrated[5,6] but no adequate scheme for converting metabolic energy into water transport has been suggested.

In recent years increasing emphasis has been placed on active transport of ions, and possibly of water itself as a means of reaching the homeostatically controlled but unbalanced states typically found in biological systems.[7] The concept of active transport has received major impetus from the experiments of Ussing and co-workers who chiefly utilized frog skin.[8] Ussing suspended frog skin between two cells containing identical solutions and was able to show a sizable potential difference between the two sides of the skin. Further, when an equal and opposite potential was applied to the cell, a current could be measured which was sustained for many hours. Later, by means of double labeling with $Na^{22}$ and $Na^{24}$ it was shown that all of the current could be accounted for by transport of sodium ion. Metabolic inhibitors or removal of sodium abolished the potential and the current. This is considered reasonable proof that the ability of frog skin to concentrate sodium ion from dilute external solutions is due to active transport.

In the case of the cornea it has been suggested that active transport of inorganic ions might well be the primary mechanism of dehydration, the water molecules following because of osmotic forces. That this might be demonstrated by a technique similar to Ussing's seemed likely to us. The necessary apparatus and early experimental results obtained are the subject of the present paper.

## EXPERIMENTAL

### MATERIAL

Beef eyes were received from the slaughter house within one to two hours after the death of the animal. No chilling was used, for eyes kept on ice for the period of trans-

* From The Laboratory for Research in Ophthalmology, Western Reserve University and Ophthalmology Service, Department of Surgery, University Hospitals of Cleveland. Supported in part by grants from the National Institutes of Health (B-30) and the Ohio Lions Clubs. A preliminary report of this work was presented before the East Central Section, Association for Research in Ophthalmology, Detroit, Michigan, January 7, 1957.

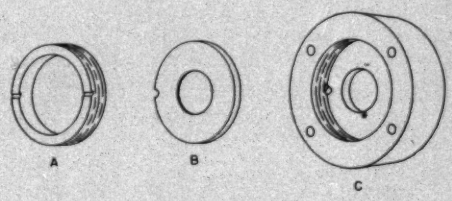


Fig. 1 (Potts and Modrell). Lucite holder. (A) Lucite screw which presses on: (B) Keyed pressure plate which holds cornea in: (C) Holder body.

portation showed a not unexpected alteration of response. Controls run at the slaughter house (five to 15 min. after death) gave only slightly higher maximum potential than corneas run in the laboratory.

The eyes were cut in half at the equator and the lens removed. Iris plus ciliary body was then stripped from the anterior segment, and the remaining cornea plus scleral ring was utilized for the experiment.

The solution bathing the corneas in all experiments was Krebs-Ringer-bicarbonate in which the total osmotic concentration had been adjusted to be equivalent to 1.35-percent sodium chloride.

## Apparatus

The anterior segment was then placed in a lucite holder designed by us (fig. 1). The holder allows a tight seal to be maintained by pressure on the scleral ring alone. The holder is then placed between two identical half cells of lucite (fig. 2) and the assembly bolted together. The remaining features are

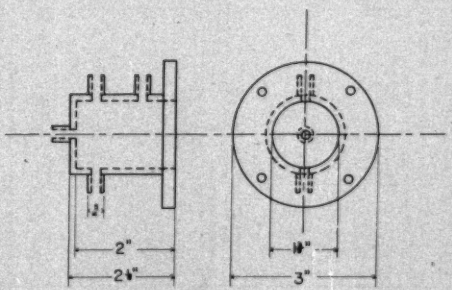


Fig. 2 (Potts and Modrell). Lucite half-cell.

adaptations of the apparatus of Ussing and Zerahn[9] (fig. 3). When the two half cells are filled with identical solution the cornea acts as the sole membrane between the two cells and, if two identical nonpolarizable electrodes (fig. 3-C) are connected to each half cell, any potential difference between them must represent corneal activity. In practise, potential differences are measured on a chopper-type DC amplifier of high sensitivity and excellent stability* (fig. 3-J) and recorded on an ink-writing galvanometer.

Further features of the apparatus are an air lift type stirrer (E), which also serves as a gassing device, to equilibrate the solutions with water-saturated air, water-saturated oxygen, or nitrogen bubbled through Fieser's solution in a bubble tower (H). There is an additional entrance to each half cell through which other salt bridges may be inserted in order to apply a short circuiting potential and measure the current driven by the transcorneal potential. In the bottom of each half cell is a sump drain through which the solutions may be emptied rapidly (D), so that alternative solutions may be substituted.

A simple photometer (fig. 4) has been constructed to allow periodic measurement of corneal light transmission without removal of the cornea from its holder. The holder can be bolted into place in the instrument over a plastic plate and rubber gasket, forming a gas-tight chamber and allowing the cornea to be inflated to a fixed pressure. This pressure (ca 325 mm. $H_2O$ = 25 mm. Hg) allows accurate and reproducible measurements of optical density.

Where water content is to be determined a plug of fixed size is cut from the cornea at the end of the experiment by a sharp stainless steel trephine chucked in a drill press. Wet weight compared to dry weight after constancy is reached in an 110°C. oven, gives water content.

* See appendix for details of equipment.

TABLE 1

A. CORNEAL-POTENTIALS AND OPTICAL DENSITY

| Experiment | Duration (min.) | Potential, mV(e p i) | | Percent Transmission | | |
|---|---|---|---|---|---|---|
| | | Initial | Max. | Initial | Final | Change |
| Cornea (1) Control | 105 | −0.54 | −1.25 | 91 | 85 | − 6 |
| Cornea (2)* Epithelium Removed | 83 | +0.120 | +0.120 | 87 | 74 | −13 |
| Cornea (3)* Endothelium Removed | 83 | −0.41 | −0.76 | 74 | 40 | −34 |

B. EFFECT OF DAMAGE TO LIMITING LAYERS IN THE SAME CORNEA

| Time (min.) | 0 | 94 | 110 | 180 | 185 | 230 |
|---|---|---|---|---|---|---|
| Potential (mV) | −1.26 | −1.32* | −0.80 | −1.20† | −0.24 | −0.24 |

* Endothelium destroyed.
† Epithelium destroyed.

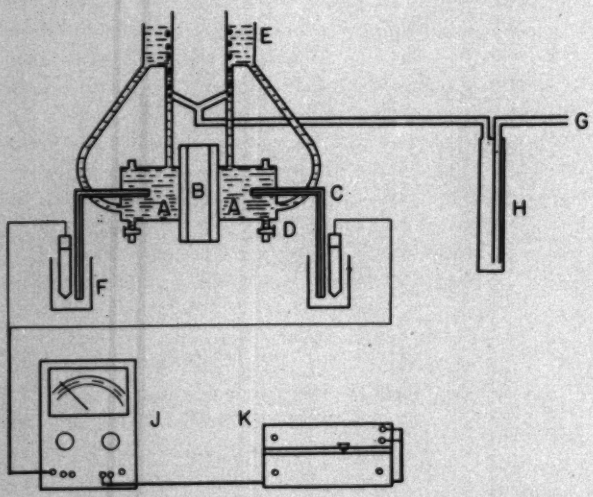


Fig. 3 (Potts and Modrell). Diagram of experimental arrangement. (A) Half-cells of Figure 2, containing medium. (B) Cornea in holder (fig. 1). (C) Salt bridge. (D) Sump drain. (E) Air-lift stirrer. (F) Electrode and salt bridge in vessel of saturated KCl. (G) Gas line. (H) Gas scrubber. (J) Chopper-type amplifier. (K) Recorder.

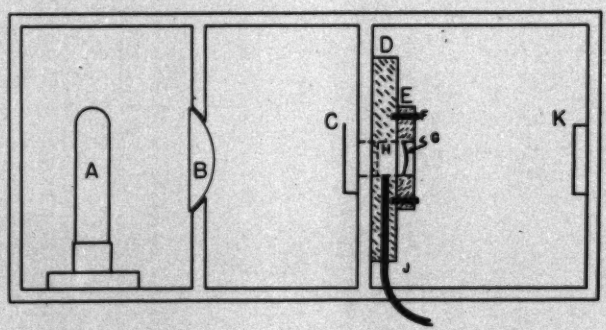


Fig. 4 (Potts and Modrell). Photometer for measuring corneal light transmission (see appendix). (A) Projection bulb. (B) Condensing lens. (C) Filter holder. (D) Pressure-tight luctie chamber. (E) Cornea holder with cornea (G) in place. (F) Bolts for holder. (H) Chamber for inflation. (J) Tubing to manometer (not shown). (K) Photocell.

## Results

*Typical experiment.* Figure 5 shows two typical experimental records. Record A is that of a normal cornea followed for a total period of 10 hours. Only the first four and one-half hours of the experiment are shown. The initial potential is −1.10 mv. rising to a maximum of −2.25 mv. in 45 min. and gradually falling over a further 9.25 hours to −0.75 mv. (epithelial side negative). The interruptions of the trace returning it to the baseline are checks for electrode drift and zeroing the system. At no time did electrode difference excede five percent of the recorded potential.

This typical trace has been repeatable many times. However, initial potential, time to reach maximum, absolute value of maximum, and rate of decay all vary considerably from eye to eye in a manner not totally predictable. Virtually all maximum values lie between 0.6 and 2.2 mv. and rise times from one-half to one and one-half hours. The initial potentials appear to be lower, the longer the time from the death of the animal, but the final maximum attained appears not to be affected by this condition.

Further, if the potential is short circuited out by an equal and opposite voltage, a current will flow in the cell. In one typical experiment where the potential was 1.15 mv. the current was 10.5/μA and was sustained for at least four hours, falling to 8.5/μA at the end of this time.

If the solutions are drained from each side of the cell, the solutions interchanged, and the cells refilled, there is no change in the potential difference observed nor its sign. This may be taken as evidence that the potential difference does not arise as a result of concentration change due to diffusion or some other passive process, but is a function of the living corneal membrane.

*Inhibition.* To investigate the biologic

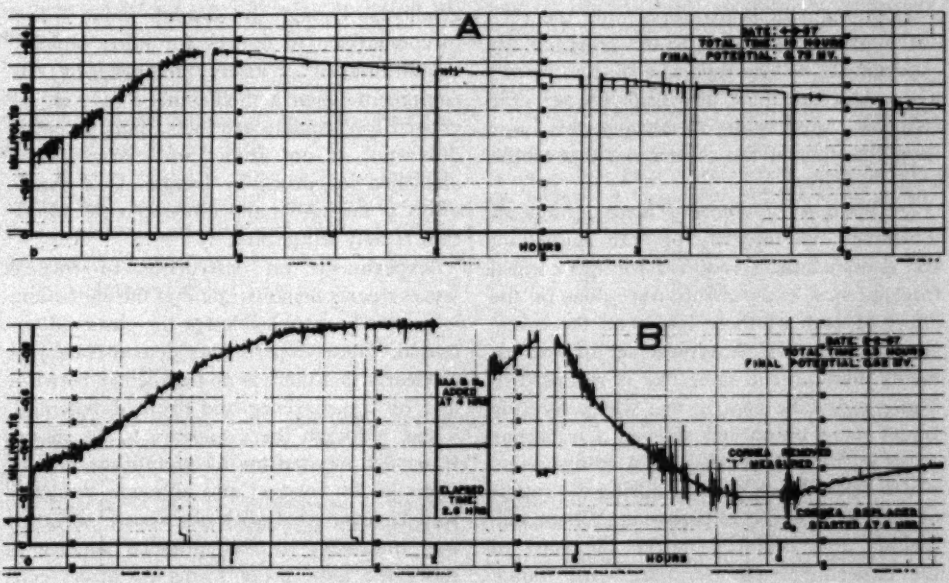


Fig. 5 (Potts and Modrell). Two typical records of the transcorneal potential. (A) Control. (B) Absence of oxygen.

origin of the potential a series of inhibitors were utilized. The one definitive result was obtained with oxygen lack. When the solution was stirred with nitrogen from which the last traces of oxygen had been removed there was a latent period of approximately 45 minutes. After this time the potential fell precipitously from 0.8 mv. to less than one fourth of this value (fig. 5-B). Aeration with oxygen restored the potential to 75 percent of its original value. In view of the physiologic fall of potential shown above this may be considered 100-percent restoration.

There was no inhibition with iodoacetate alone, or azide. Dinitrophenol gave a temporary inhibition which was soon overcome.

A further control experiment consisted in using the posterior segment of sclera instead of the anterior corneoscleral preparation in the cell. This preparation gave no measurable potential. From this it may be logically concluded that in the case of the anterior segment preparation, only the corneal portion contributes to the potential. In experiments to be reported in the future where ion flux must be calculated per unit area of transferring membrane this area should be that of the cornea, not that of the holder aperture.

*Destruction of limiting layers.* Since it is known that integrity of both epithelium and endothelium is required for full corneal transparency, experiments were done on the effect of each of these layers on the transcorneal potential. A typical set of experiments done on the same day is recorded in Table 1-A. The control eye where limiting layers were untouched showed a minimum potential of −1.18 mv. and a transmission which fell only six percent during the course of the 105-minute experiment. Where epithelium was removed before the start of the experiment (by scraping with round-bellied scalpel blade) the potential never did become negative and final transmission was down 13 percent. In accord with clinical observation the greatest drop in transmission (34 percent) occurred in the cornea where endothelium was removed. However potential in this cornea did attain −0.76 mv.

These results are amply confirmed on repetition, and by a slightly different type of experiment as shown in Table 1-B. Here an intact cornea was run until it reached maximum potential—in this case −1.32 mv. at 94 minutes. At this point the endothelium was scraped. Though the potential dropped (−0.80 mv.) this was only temporary and after 180 minutes the potential had recovered to −1.20 mv. On destroying epithelium, however, the potential fell to −0.24 mv. and remained there for at least 45 minutes when the experiment was concluded. As before, the major drop in light transmission occurred on endothelial damage.

## Discussion

From the foregoing it seems a valid conclusion that a transcorneal potential does, indeed, exist as the result of biologic activity and independent of concentration gradients. Though the absolute value is low in comparison to figures for other biologic membranes, it is easily measured by our equipment when a modicum of care is exercised to eliminate artefacts. This potential difference is not found in sclera, and is abolished in cornea by oxygen lack. Azide effect is negligible and dinitrophenol inhibition is only temporary.

Experiments on destruction of corneal layers clearly demonstrate that the epithelium is primarily responsible for the observed potential. These experiments demonstrate just as clearly that there is no parallelism between loss of transparency and drop of potential. Thus, although there appears to be an ion transport mechanism of significant dimensions in the cornea; and although this may well be connected with previously observed impermeability of the epithelial surface to inorganic ions, there seems no direct connection between this mechanism and corneal transparency. Further, since destruction of the layer chiefly affecting transparency, that is, endothelium, has little effect on the potential, one might conclude that ion transport

has little to do with corneal transparency. If, indeed, the endothelial role is one of maintaining dehydration one would be forced to say that such dehydration must be accomplished without net ion transport—possibly by direct transport of water.

Future work will deal in greater detail with the short circuit current and the ions responsible for carrying the current. The role of endothelium will be examined further with the help of tritiated water. A possible mechanism for the direct transport of water will be looked for.

## Summary

1. A potential may be measured across the isolated beef cornea when both sides are bathed in an identical solution of Krebs-Ringer-bicarbonate. This potential ranges from 0.6 to 2.2 mv. with the epithelial surface the more negative.

2. This potential when short circuited by an equal and opposite potential can drive a current of 10 ma. or more for a period of hours.

3. This potential is not a property of sclera.

4. The potential may be abolished or nearly so by oxygen lack.

5. The potential is a property of corneal epithelium, and there is no direct connection between potential and corneal transparency.

*2065 Adelbert Road (6).*

## Appendix on equipment

1. Microammeter-voltmeter. This instrument is Kay-Lab Model 203. It is chopper stabilized and provides a maximum gain of 80 D.B. on the lowest meter range. Output on all ranges is one-volt full scale, more than adequate to drive most recorders. Input impedance of the range 0 to 1 millivolt is approximately 10 megohms. Stability is excellent with no drift for 10 hours after a 30-minute warm-up period.

2. Recorder. This is a Varian Grapher Model G-10. Our instrument is driven at a fixed speed of 4 inches per hour. The recorder will give full scale deflection (linear writing on 5-inch chart paper) with 100 microvolts input to the Kay-Lab amplifier (its most sensitive range).

3. Electrodes. For measurement of potential these are identical Beckman Calomel Electrodes #B-4970 dipping into 200 cc. of saturated KCl and connected by capillary salt bridges of Agar-KCl (saturated) to their respective half-cells.

During the experimental period the corneal cell, the calomel electrodes and salt bridges are enclosed in a shield of copper screen grounded directly to earth. The levels of amplification at which we work require some attention to extraneous electrical noise. Such remedies must be found empirically to fit local conditions.

4. Photometer. This instrument built by us uses as light source an 18 ampere, six volt projection bulb GE #18A/T10-6V operated at a constant voltage of approximately 4.5 volts. The sensing element is a barrier-layer photocell GE #PV-1. Current output from the cell is read on the Kay-Lab meter described in paragraph 1.

The photometer was calibrated by the use of Wratten neutral density filters (Eastman Kodak #96) over the range 0.1 to 3.0 log units. Density vs log current gave an excellent straight line plot and allowed us to calculate density from an empirical formula:

$$d = \frac{\log \mu a}{-1.09} + 1.84.$$

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## Discussion

Dr. D. Venkat Reddy: After listening to the excellent presentation by Dr. Harris, I have decided to forego some of the remarks I was going to make, because I am sure that after his lecture my remarks would be repetitious.

Dr. Harris has presented an impressive amount of evidence to show that there is some sort of active transport mechanism for the regulation of the dehydration mechanism in the cornea. I would like to congratulate Dr. Potts for so successfully employing the techniques developed by Dr. Ussing in the measurement of transcorneal potentials. I believe this is perhaps the first attempt to show directly that there is a potential across the cornea, and in this fashion one can demonstrate that there is an active transport mechanism.

Those of us who have attempted to measure bioelectric potentials realize the meticulous care needed to record potentials of the order of a millivolt which Dr. Potts has measured here. It is only in capable hands that one can be quite certain that these potentials do exist. I have no doubt that Dr. Potts has succeeded in showing that they are there.

The fact that this potential can be abolished by anoxia and also reversibly inhibited by dinitrophenol suggests that high energy phosphate bonds, probably ATP, are involved in maintaining this potential and possibly the active transport mechanism.

A further evidence for the existence of this mechanism has been suggested because of the measurement of the short-circuited current which was approximately 10 microamperes. I have just made some rough calculations and this would amount to about 18 to 20 millicoulombs per hour per square centimeter, assuming the area of the cornea was about four square centimeters. This represents a considerable ion transport almost one-tenth of the amount observed by Dr. Ussing in frog skin.

However, the demonstration alone of the existence of a short-circuited current need not be taken as evidence for the size of this amount of ion transport. Of course, the contemplated studies of Dr. Potts will probably establish (when he has had an opportunity to measure the flux of the sodium ion and possibly the tritiated water) that this short-circuited current is actually equivalent to the ion being transported.

Also, these projected studies may indicate whether there is an ion pump or, as Dr. Harris suggested, possibly a water pump.

I would like to thank the authors for giving me the opportunity to discuss this paper.

Dr. Albert M. Potts (closing): I would like to thank Dr. Reddy for his comments.

It is true that both instrumentation and technique in the hands of Mr. Modrell, who is here, are responsible for the reproducibility of these results. Without the particular apparatus which we used, I doubt whether our measurements would have been successful. Gadgetry in this particular case does play an important role.

I did not mention the dinitrophenol partly because of lack of time and partly because I don't know how to explain its effect. The dinitrophenol inhibition causes a hesitation in the rise of the transcorneal potential, but the cornea apparently can overcome the inhibition of any sensible amount of dinitrophenol and keep on rising after it stays on the plateau for a little while.

As long as we do not have to account for transport of water against a leaky membrane, perhaps the amount of current that is involved in the transcorneal potential is not too significant here; if we are interested in water transport and its connection with transparency we are going to have to measure transport of water molecules rather than transport of ions.

# INCORPORATION OF INORGANIC SULFATE-$S^{35}$ INTO SULFATED MUCOPOLYSACCHARIDES OF CORNEA IN VITRO*

BERNARD WORTMAN, PH.D., AND JACK L. STROMINGER, M.D.
*Saint Louis, Missouri*

Of the mucopolysaccharides known at the present time,[1] the cornea contains keratosulfate, chondroitin, and chondroitin sulfate. Keratosulfate,[2] the principal acid mucopolysaccharide of the cornea, is also present in some connective tissues. Chondroitin[3] is found only in cornea. Of the three forms of chondroitin sulfate (designated A, B, and C) only A is found in cornea.[1] The ratio of these three compounds in beef cornea has been reported[2] as approximately 2:1:1. Preliminary to further enzyme studies, we were interested in establishing whether the sulfated mucopolysaccharides of cornea, especially keratosulfate, are in an active metabolic state. For this purpose, the incorporation of inorganic sulfate-$S^{35}$ into the acid mucopolysaccharides of cornea in vitro has been investigated.

## MATERIALS AND METHODS

### INCUBATION OF CORNEA AND CARTILAGE WITH INORGANIC SULFATE-$S^{35}$

Adult rabbit cornea and costal cartilage and beef cornea were used. Whole cornea or pieces thereof and slices of costal cartilage (0.5 to 1.0 mm. in thickness) were incubated in Difco TC 199 tissue culture medium supplemented with $H_2S^{35}O_4$ (carrier free, obtained from Oak Ridge National Laboratories and neutralized), 50 units of penicillin and 6 μg. of streptomycin per ml. of culture medium. The antibiotics were added to inhibit bacterial growth. Incubation mixtures were agitated vigorously at 38°C. for 18 hours or longer. Controls were heated at 100°C. for 10 minutes prior to incubation or stored at −20°C. without incubation. At the end of an incubation period, incubation mixtures were heated for 10 minutes at 100°C., washed three times with water, suspended in 0.1 M $Na_2SO_4$ for three hours at 38°C., and again washed three times with water. The procedure was convenient for removal of inorganic sulfate-$S^{35}$ from cornea but inadequate for cartilage.

*Radioactivity measurements.* Two procedures were used to prepare tissue samples for radioactivity detection. Samples were counted to one or two percent error in a windowless, gas-flow counter (Nuclear Instrument Co., Chicago, Illinois). Procedure A: The tissue was hydrolyzed in 1.0 ml. of concentrated HCl at 100°C. for three hours; 1.0 ml. of 0.05 *M* $Na_2SO_4$ and 1.0 ml. of 0.1 *M* $BaCl_2$ were then added. The $BaSO_4$ precipitate was centrifuged, resuspended in a few drops of 1:10,000 Zephiran Cl, transferred to a circle of 4.0 $cm.^2$ area on an aluminum planchette, dried and counted at infinite thickness. Procedure B: Acid mucopolysaccharides were isolated as described by Meyer et al.[2] Purification was carried up to the Lloyd's reagent extraction. The isolated acid mucopolysaccharides were taken up in water. A small aliquot (3 μl.) was plated and counted.

*Hyaluronidase digestion of polysaccharides.* Samples of 1.16 mg. of chondroitin sulfate from bovine nasal cartilage (General Biochemical Corporation, Cleveland, Ohio) and 0.94 mg. of keratosulfate† were incubated for 24 hours at 38°C. with 142 units of testicular hyaluronidase (Wyeth Labora-

* From the Departments of Ophthalmology and Pharmacology, Washington University School of Medicine. This investigation was supported in part by research grants B-621, from the National Institute of Neurological Diseases and Blindness and A-1158(c) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

† We wish to thank Dr. Karl Meyer and Dr. Alfred Linker for the keratosulfate used in this study as a reference standard.

tories, 1500 units/mg.*). The hyaluronidase did not contain any metachromatic material. Samples of isolated corneal and scleral mucopolysaccharides were similarly treated.

According to Meyer et al.[1] chondroitin and chondroitin sulfates A and C are hydrolyzed by this enzyme, while chondroitin sulfate B and keratosulfate are not.

*Paper electrophoresis.* Electrophoresis of acid mucopolysaccharides was conducted in 0.05 M phosphate buffer at pH 7.0 on Whatman No. 1 paper at a voltage gradient of approximately 20 V/cm. for 60 minutes in an apparatus as described by Markham and Smith.[4] The basis of electrophoretic separation of chondroitin sulfate and keratosulfate[5] is their ionic charge. At pH 7.0 chondroitin sulfate carries two negative charges (sulfate and glucuronic acid) while keratosulfate has only one (sulfate). However, chondroitin sulfate does not move at twice the rate of keratosulfate. Acid mucopolysaccharides were detected by metachromatic staining.[5,6] The paper was immersed five minutes in 0.1 percent toluidine blue in 50 percent ethyl alcohol and washed in 50 percent ethyl alcohol to lighten the background. Radioautographs of the electrophoretic strips were produced prior to staining by allowing the Whatman paper to remain in contact with Kodak blue brand radiographic film for approximately one month.

## Results

*Adequacy of controls.* Contamination of the tissue by inorganic sulfate-$S^{35}$ is not as great a problem in cornea as in cartilage. Inorganic sulfate-$S^{35}$ bound by cartilage is not readily removed by dialysis and more extensive washing must be employed.[7] Heat denaturation increased this binding. However, dialysis removed practically all inorganic sulfate-$S^{35}$ from cornea.[8,9] The procedure employed in this study removed essentially all radioactivity from corneal controls, whether heat treated or stored at −20°C.

* Supplied by Dr. Luis Glaser of the Department of Biochemistry.

TABLE 1

UPTAKE OF INORGANIC SULFATE-$S^{35}$ BY RABBIT CORNEA AND CARTILAGE IN VITRO

Pieces of cornea or slices of cartilage, 50 mg. wet weight, were incubated in 1.0 ml. TC 199 medium containing $3\times10^6$ c.p.m. inorganic sulfate-$S^{35}$. $BaSO_4$ was isolated and counted at infinite thickness. Values are the mean of two or three determinations. The results are expressed as c.p.m./mg. wet tissue.

| Incubation | Cornea | Cartilage |
|---|---|---|
| Control: | | |
| a) −20° 18 hours | 0.90 | 6.9 |
| b) 100° 10 minutes, then 38° 18 hours | 0.94 | 23.4 |
| Experimental: | | |
| a) 38° 18 hours, then 100° 10 minutes | 31.4 | 60.5 |

(table 1). Cartilage controls were high in radioactivity, and the heat-treated tissue contained almost three times as much radioactivity as tissue stored at −20°C. (table 1).

*Inorganic sulfate-$S^{35}$ incorporation into cornea.* Rabbit cornea incorporated inorganic sulfate-$S^{35}$ into an organic form at a linear rate for approximately 18 hours (fig. 1 and table 1).

For comparison similar experiments were performed with rabbit costal cartilage slices. The large scatter shown in Figure 2 was probably due to incomplete removal of inorganic sulfate-$S^{35}$. An exact comparison is not feasible, but it is evident that the uptake by cornea and by cartilage were of the same order of magnitude. These figures also illustrate an advantage of using cornea in this type of study.

*Localization of incorporated sulfate-$S^{35}$.* Acid mucopolysaccharides isolated from rabbit and beef corneas contained significantly more radioactive material than did heat-treated controls (table 2). The mucopolysaccharides in this fraction (CP) had the same electrophoretic mobility as authentic keratosulfate (KS) (fig. 3). By contrast sclera contained acid mucopolysaccharides (SP) with an electrophoretic mobility of chondroitin sulfate (fig. 3). The absence from these corneas of a compound with the electrophoretic mobility of chondroitin sulfate is also noteworthy.

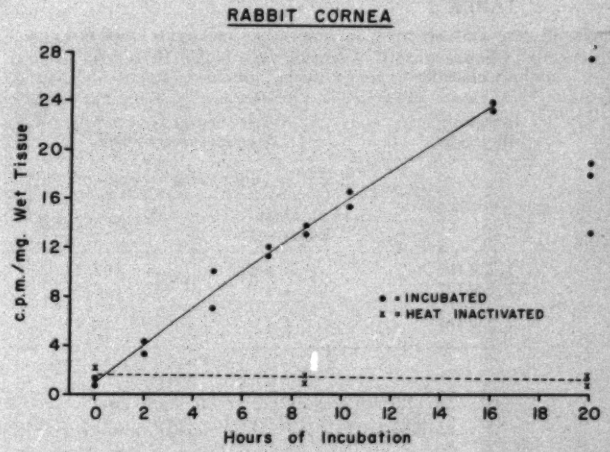


Fig. 1 (Wortman and Stromінger). Uptake of inorganic sulfate-$S^{35}$ by rabbit cornea in vitro. Whole cornea or pieces were incubated at 38°C. Controls were heat inactivated prior to incubation. Each point represents approximately 50 mg. wet tissue in 1.0 ml. TC 199 supplemented with $2 \times 10^6$ c.p.m. inorganic sulfate-$S^{35}$. At 20 hours the culture medium was highly acid, as indicated by the presence of phenol red in TC 199 medium $BaSO_4$ was isolated from the acid hydrolyzed tissue and counted at infinite thickness.

A radioautograph of an electrophoretic strip indicated correspondence between the corneal mucopolysaccharides (CP) and the location of organic sulfate-$S^{35}$ (fig. 4). A very small amount of inorganic sulfate-$S^{35}$ contaminated the polysaccharide fraction and appeared as a fast moving, nonmetachromatic, radioactive spot.

Digestion with testicular hyaluronidase was carried out to define further the nature of the isolated mucopolysaccharides. Hyaluronidase caused a loss of practically all metachromasia from chondroitin sulfate and had no effect on keratosulfate (fig. 5). A major portion of the sulfate-$S^{35}$ in the corneal mucopolysaccharide fraction was resistant to testicular hyaluronidase (CP + H, figs. 3 and 4). A small portion of the corneal fraction was degraded and appeared as a metachromatic and radioactive material with a slightly higher electrophoretic mobility than keratosulfate. The scleral fraction was digested in a manner similar to chondroitin sulfate A with loss of metachromasia and appearance of a fraction of higher electrophoretic mobility (SP + H, fig. 3).

## Discussion

Rabbit and beef corneas have been shown to be capable of in vitro incorporation of inorganic sulfate-$S^{35}$ at a rate approximately the same as costal cartilage. Acid mucopoly-

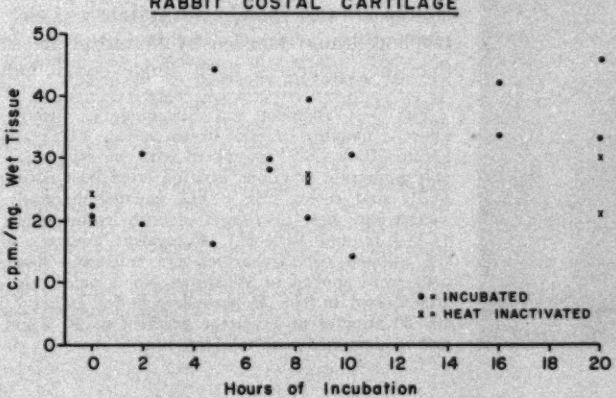


Fig. 2 (Wortman and Stromінger). Uptake of inorganic sulfate-$S^{35}$ by rabbit costal cartilage in vitro. Slices of rabbit costal cartilage (0.5 to 1.0 mm. in thickness) were incubated at 38°C. Controls were heat inactivated prior to incubation. Each point represents approximately 50 mg. wet tissue in 1.0 ml. TC199 supplemented with $2 \times 10^6$ c.p.m. inorganic sulfate-$S^{35}$. $BaSO_4$ was isolated from the acid hydrolyzed tissue and counted at infinite thickness.

TABLE 2

INCORPORATION OF INORGANIC SULFATE-$S^{35}$ INTO ACID MUCOPOLYSACCHARIDES ISOLATED FROM CORNEA

Tissues were incubated in TC 199 medium for 18 hours at 38°C. Controls were boiled 10 minutes prior to incubation. Isolated acid mucopolysaccharides were counted at infinite thinness.

| Animal | Weight of Tissue | Inorganic Sulfate-$S^{35}$ | Radioactivity in Isolated Acid Mucopolysaccharides | |
|---|---|---|---|---|
| | | | c.p.m./mg. tissue | |
| | mg./ml. medium | c.p.m./ml. medium | Heat Inactivated | Experimental |
| Rabbit No. 1 | 50 | $1.5\times10^6$ | 0.6 | 102.1 |
| Rabbit No. 2 | 50 | $1.5\times10^6$ | 4.1 | 138.6 |
| Beef | 100 | $1.1\times10^6$ | 4.7 | 36.1 |

saccharides isolated from such corneas contained sulfate-$S^{35}$. The major portion of this fraction appears to be keratosulfate on the basis of its electrophoretic mobility and resistance to hyaluronidase. The exact nature of the smaller portion, digested by hyaluronidase, is uncertain, and several possibilities suggest themselves. It might be chondroitin and some chondroitin sulfate of a low degree of sulfation. Chondroitin with a single negative charge would be expected to have the same electrophoretic mobility as keratosulfate and a low sulfate content would not appreciably effect electrophoretic mobility. It may be noted that the chondroitin described by Davidson and Meyer[3] contained a small amount of sulfate. They indicated that it was not possible to conclusively prove the existence of chondroitin entirely free of sulfate. The possibility was also considered that a fully sulfated chondroitin sulfate was electrophoretically retarded by keratosulfate so that they appeared as a single compound.

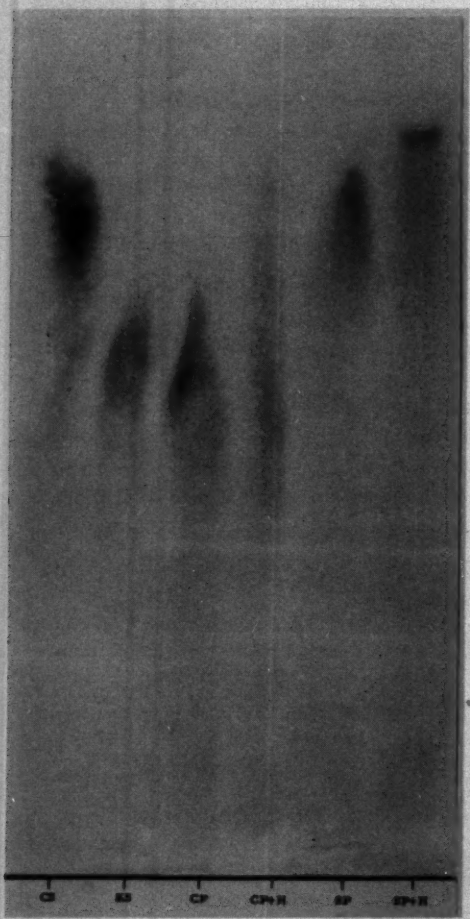


Fig. 3 (Wortman and Strominger). Electrophoretic mobility of chondroitin sulfate (CS) and keratosulfate (KS) compared with the acid mucopolysaccharide fractions isolated from beef cornea (CP) and sclera (SP). The isolated mucopolysaccharides were also digested with hyaluronidase (CP + H and SP + H). Equivalent amounts of the isolated polysaccharides and reference standards were applied to Whatman No. 1 paper, electrophoresed in 0.05 *M* phosphate buffer at pH 7.0 for 60 minutes at a voltage gradient of 20 V/cm. and then stained with toluidine blue.

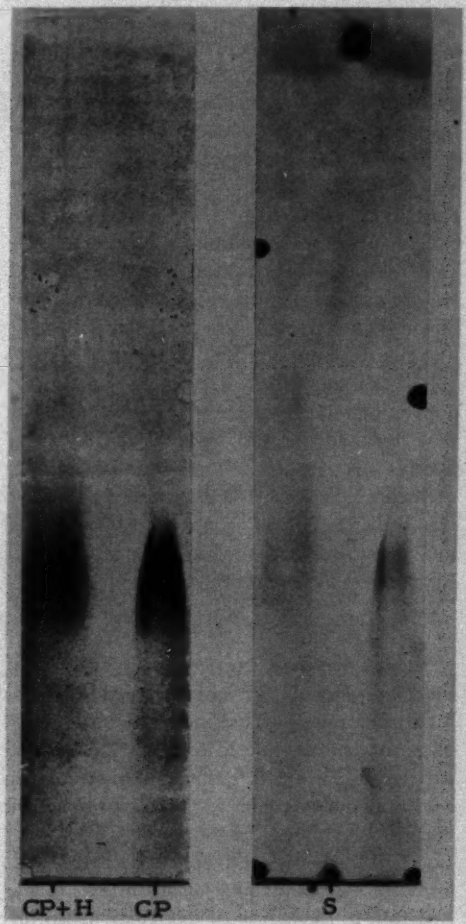


Fig. 4 (Wortman and Strominger). Comparison of the electrophoretic mobility of the metachromatic (toluidine blue staining) material and of the organic sulfate-$S^{35}$ in mucopolysaccharides isolated from incubated beef cornea (CP). The figure at the left is the electrophoretic strip stained with toluidine blue and that at the right is the radioautograph of this strip. The black spots at the base and margins of the radioautograph are markers for orientation of the film with the paper. A small amount of radioactivity in the mucopolysaccharides corresponded in electrophoretic mobility to inorganic sulfate-$S^{35}$ (S, the dark spot at the top). The radioactive corneal polysaccharides were also digested with hyaluronidase (CP + H).

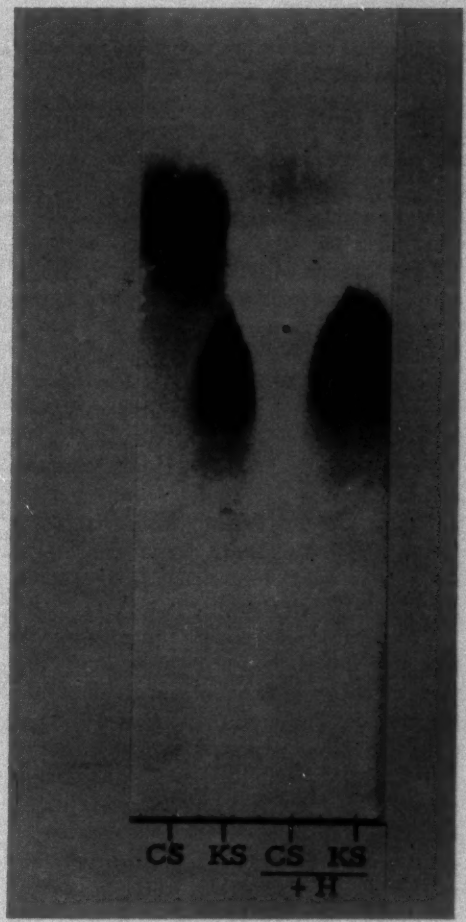


Fig. 5 (Wortman and Strominger). The effect of testicular hyaluronidase on mucopolysaccharides as demonstrated by paper electrophoresis. Approximately 1.0 mg. samples of chondroitin sulfate (CS) and keratosulfate (KS) were incubated for 24 hours at 38° with 142 units of hyaluronidase (H). The polysaccharides were applied to Whatman No. 1 paper and the electrophoresis conducted in 0.05 *M* phosphate buffer at pH 7 for 60 minutes at a voltage gradient of 20 V/cm. The paper was stained with toluidine blue.

However, when chondroitin sulfate and keratosulfate were applied to paper in approximately 1:2 mixture, a visible separation with some tailing was observed. A third possibility might be that corneal chondroitin sulfate has a higher molecular weight than our reference standard. It would be possible that a much larger molecular size would reduce the electrophoretic mobility of corneal chondroitin sulfate to that of keratosulfate.

Incorporation of inorganic sulfate-$S^{35}$ into cartilage[7,8] and skin[9] in vitro has been shown previously. Concurrently with this study, Dohlman et al.[10,11] reported inorganic sulfate-$S^{35}$ uptake by rat and rabbit corneas in vitro but did not demonstrate its localization in isolated acid mucopolysaccharides. Also, Roden[12] recently has shown that inorganic sulfate-$S^{35}$ was incorporated into nucleus pulposus. In this case radioactivity was found in both chondroitin sulfate and keratosulfate-like fractions isolated from that tissue.

Very little is known concerning the mechanisms of mucopolysaccharide synthesis and particularly little about sulfated mucopolysaccharides. The recent description[13] of uridine diphosphate acetylgalactosamine sulfate (an activated form of acetylgalactosamine sulfate) suggests that in some cases sulfation may occur prior to incorporation of sugars into polysaccharides. However, Duda and Pogell[14] detected no sulfated uridine nucleotides in beef corneal epithelium and we failed to find such a compound in whole beef cornea (unpublished data). However, this failure might result only from a low steady state concentration of such a hypothetical intermediate compound. Whether nucleotides or unsulfated polysaccharides are the primary acceptors of sulfate in the biosynthesis of sulfated mucopolysaccharides by cornea and other tissues is an open question and indeed both mechanisms and others might operate in different cases. However, it seems likely that, whatever the nature of the intermediate compounds, sulfate activation by ATP, as occurs for the synthesis of other sulfated compounds in liver[15,16] and in yeast,[17] is also the primary mechanism of activation of sulfate for polysaccharide formation.

Physiologic observations should be evaluated with the newer knowledge of corneal and scleral polysaccharides. When the anterior chamber angle was perfused with hyaluronidase from various sources, Bárány[18] found that regardless of the enzyme source, approximately 50-percent decrease in outflow resistance could be achieved in some species. It is an interesting speculation (which could be tested experimentally) that the trabeculae may contain a mixture of mucopolysaccharides which are in part not hydrolyzed by hyaluronidase.

## Summary

Rabbit and beef corneas were capable of inorganic sulfate-$S^{35}$ uptake in vitro. Rabbit cornea incorporated inorganic sulfate-$S^{35}$ at a rate comparable to that of rabbit costal cartilage. Sulfate-$S^{35}$ was demonstrated in acid mucopolysaccharides isolated from such corneas. A major fraction of this preparation had the same electrophoretic mobility as keratosulfate, and, like keratosulfate, it was resistant to the action of testicular hyaluronidase. A smaller portion, possibly chondroitin with a low degree of sulfation, was hydrolyzed by hyaluronidase.

In contrast to cornea, beef sclera contained an acid mucopolysaccharide with the electrophoretic mobility of chondroitin sulfate, and, like chondroitin sulfates A or C, it was degraded by testicular hyaluronidase.

*640 South Kingshighway (10).*

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## Discussion

Dr. E. Albert Zeller (Chicago): This paper is an excellent example of the application of modern biochemical and biophysical methods to the study of the eye. Dr. Karl Meyer should be pleased by how well the field is tended which he opened many years ago.

While the results speak for themselves, I still would like to ask a few questions. In chondroitin sulfate of cartilage, as well as in sulfolipids of the nervous system of normal animals, the incorporation of labelled sulfate turned out to be irreversible. Do the authors have any information regarding the reversibility of the incorporation of $S^{35}$ into sulfated mucopolysaccharides of the cornea?

The second question pertains to the use of streptomycin to avoid bacterial contamination. Did you test the possibility that streptomycin does not interfere with the incorporation of sulfate? After all, the structures of the mucopolysaccharides and of streptomycin show certain similarity.

Third, Dr. Wortman stated that testis hyaluronidase does not attack some of the mucopolysaccharides, such as keratosulfate. Is that true of snake venom hyaluronidase, which is similar but not identical?

Dr. Bernard Wortman (closing):

Data presented do not necessarily measure the turnover of sulfated mucopolysaccharides. Whether we are dealing with the synthesis of new mucopolysaccharides or only with the exchange of sulfate is not known. Published evidence suggests that in skin sulfate uptake represents the synthesis of new sulfated mucopolysaccharide. We cannot comment on the reversibility of sulfate incorporation or on the effect of various antibiotics on the sulfation process until more is learned about the mechanisms involved. The antibiotics of choice have been penicillin and streptomycin. Aureomycin was found to be inhibitory to sulfate uptake by cornea. Snake venom hyaluronidase was not tested. Testicular hyaluronidase was used to define further the corneal mucopolysaccharides because its action on various chondroitin sulfates and keratosulfate has been previously described and so is most useful for the purposes of this study.

I hope that answers the questions. I wish to thank Dr. Zeller for his discussion.

# IN VIVO INTRALENTICULAR STUDIES*

A. W. Vogel, M.D., and D. A. Reinoehl, M.S.
*Philadelphia, Pennsylvania*

Study of the lens has been extensive and its literature is voluminous. This is a presentation of an approach to further manipulation of the lens in the normal and altered state. As far as we can determine from the literature, it has not been extensively tried before.[1] It describes intralenticular implantation of solids, the injection of minute quantities of various liquids, and observations to date. The method of making suitable pellets and a description of the instrument used for their implantation are to be published.[2]

## I. Solid substances

### A. Method

Rabbits ranging in age from six weeks to three years and over were used. A retrobulbar injection of two-percent Novocaine is given and after 10 minutes the eye is proptosed with a muscle hook. Absolute insensitivity must be demonstrated prior to implantation. Effectiveness of the anesthesia is tested over several sectors of the globe with a Birch pick. Sensitivity as manifested by a slight retraction of the globe indicates that anesthesia is inadequate. If there has not been adequate anesthesia the animal jumps as the needle passes through the ciliary body. The resulting large subcapsular and/or nuclear opacities formed by the needle's erratic stroking through the lens substance render the specimen useless. The pellet is protected from the moisture of the conjunctival secretion by covering the globe with a piece of transparent cellophane prior to positioning the instrument. The needle guard of the Kaczurowski pellet injector is placed 2.0 mm. behind the limbus in the adult rabbit and 1.5 mm. in the young, and it is steadied with the Birch pick. The direction the needle is to travel is sighted parallel to the iris plane and toward the center of the lens (fig. 1). After the stylet has ejected the pellet the needle is withdrawn from the lens as closely as possible along the tract of entrance.

### B. Results

#### 1. *Trauma*

The trauma caused to the globe and lens is roughly proportional to the size of the needle used. Hyphemia has occurred in only 1:38 eyes implanted with a No. 27 needle, in 3:12 with a No. 24 needle, while it has occurred in 3:6 eyes in which a No. 21 needle was used. The passage through the ciliary body is usually half way between the limbus and ora serrata. Sectioning of the globes at the equator often shows a ribbon of blood at the puncture site and/or invariably a fibrin strand extending from it to the needle hole in the lens.

The entrance of the needle was at the equator in 17:42 cases, and behind the equator in 25:42 cases. Entrance posterior to the equator usually caused a posterior circular opacity 2 mm. average diameter which in the first few days postoperative had a bleblike appearance. Entrances on the equator caused average opacities of 0.5 mm. in diameter.

The needle tract is difficult to see in the otherwise normal lens. The site does not become opaque but there is evidence of disturbance as is shown by its more plastoid and wary appearance as contrasted with the usually seen luminescence and regularity of normal stroma.

The tolerance of the lens to the presence of a foreign body is shown by four eyes. Glass capillary needles were implanted into

* From the Department of Research, Wills Eye Hospital, Irving H. Leopold, M.D., Director. This work was supported by Grant B-998 from the National Institutes of Health. We wish to thank Dr. Harry Green, Department of Biochemistry, for his helpful discussions pertaining to this work, and Mr. Thomas Walsh for the photography.

Microinjector and pellet implanter made by F. J. Todd, 201 Forrest Avenue, Narberth, Pennsylvania.

Fig. 1 (Vogel and Reinoehl). Positioning of animal and instruments prior to pellet implantation. Note that the implanter is sighted along the plane of the iris and directed toward the center of the lens.

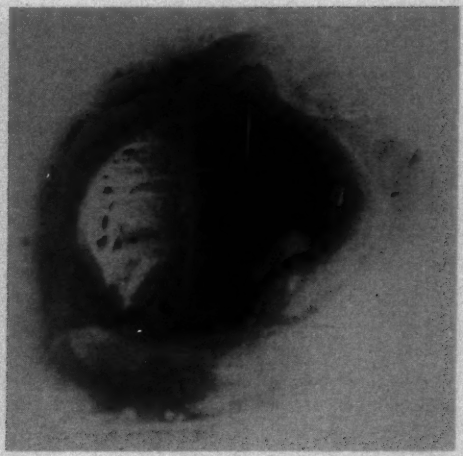

Fig. 2 (Vogel and Reinoehl). Tolerance of the interior lens matrix to the presence of a foreign body is demonstrated by the clear lens stroma surrounding a No. 32 stainless steel needle which was left in situ for 11 months.

two eyes, and No. 32 stainless steel needles were implanted into two. They were observed for one year (fig. 2). There is still no evidence of disturbance to the surrounding lens fibers and the lenticular appearance is consistent with that of normal two-year-plus old rabbits. It has been pointed out that possibly because of the relative inelasticity of the lens capsule, puncture wounds of the lens in the lower vertebrates do not lead to as extensive a change as that found in man.[8]

### 2. *Foreign protein*

a. *Procedure.* Pellets of lyophilized horse serum were implanted into one eye each of two rabbits after seven- and 14-day intervals following subcutaneous injection of 2.5 cc. of horse serum. At the time of implantation the blood serum precipitin titer was moderately to strongly positive in one animal at seven days, and both at 14 days.

In a second group horse serum was implanted into one eye each of two unsensitized animals; implantation was repeated in the contralateral eye one week later. One week after the last implantation 1.5 cc. of horse serum was given intravenously. Five months after the first pellet, a pellet of antihorse serum was implanted in one eye of both animals, while in the other eye a pellet of kaolin was implanted as a control.

b. *Appearance and results.* Both groups were followed by the slitlamp for a five-month period. Immediate examination showed a yellowish tinged cylindrical pellet approximately 0.5 mm. by 1.4 mm., lying two to three mm. inside the anterior lens capsule near the pupillary center.

A 0.1 to 0.7 mm. nebulous opacity appeared around the deposits within a week of implantation. This persisted throughout the period of observation. There were no other observable changes in the lens in the animals previously sensitized or given intravenous injection of horse serum following implantation. After four months the compactness of the pellet had diminished and there was a thinning of the individual fragments. This was not measured quantitatively. Those lenses implanted with the pellet of antihorse

serum or kaolin have remained inert to their presence.

c. *Discussion.* The surface area/volume ratio of the usual particulate or soluble protein suspensions is huge compared to that present in this deposit. The compactness and resulting small surface area of the pellet as presented by those fragments seen at ×15.5 magnification in this experiment may partially account for its inertness to the action of lenticular proteolytic enzymes during the period of observation. It is unlikely that the presence of trauma from the implantation of the foreign protein caused a release of an appreciable amount of proteolytic enzyme.

It has been indicated that the corneal epithelium is capable of antigenic response.[4] It is not inconceivable that the lens epithelium possesses this property. The absence of further opacity or precipitate within the lens substance following the above described sequence of implantations indicates that under the circumstances of the experiment the following suppositions will have to be further pursued: (1) antibody is not diffusible through the capsule, (2) this form of antigen is not diffusible through the lens matrix, (3) the lens epithelium does not produce antibody, (4) or if the antibody-antigen reaction does take place within the lens it is invisible.

### 3. *Dyes*

A dye suitable for the purpose of intralenticular implantation must be freely soluble in the intra and/or intercellular fluids, nontoxic in the workable concentrations required, and easily seen. We have screened four dyes thus far for their suitability in in vivo lens diffusion studies.

a. *Trypan blue and trypan red.* These substances are only slightly soluble in the lens stroma. Four eyes were implanted with each. The pellets were discrete and well formed on withdrawal of the needle. The average spread, during the entire observation period of three weeks, into the clear lens matrix surrounding the pellet was one mm. In two eyes an apparently preferential streaming of a thin current of faintly seen trypan red was observed to follow a suture line towards the posterior capsule, for a distance of three to four mm.

b. *Methylene blue.* This substance was implanted into one eye. The immediate postoperative appearance was a dark blue pellet one mm. in diameter surrounded by a much lighter faint blue fringe, measuring 0.06 mm. in diameter. Within 18 hours it had increased in diameter to 1.5 mm., become completely white and crystallized. There was no change in its shape or surface appearance over a four month period.

c. *Discussion.* Trypan blue, trypan red and methylene blue were unsatisfactory substances for a diffusion study. These compounds spread for only a limited distance. Methylene blue was reduced and crystallized. The trypans show binding of the main bulk of the pellet in situ with preferential streaming of a small moiety or fraction apparently along a suture track to the posterior capsule.

d. *Fluorescein sodium—normal lens.* This substance is apparently freely soluble in the lens matrix because on withdrawal of the needle a heavy green streak is immediately formed. This is in contrast to the previously described dyes which could be seen as discrete and intact pellets suspended in the interior of the lens. The postoperative appearance for the first eight hours is that of a gradually widening dark green ribbon extending from the junction of the lower and central thirds of the lens to the equator at the 12-o'clock position. As would be expected from the complicated arrangement of the fibers there was always eventual asymmetric dispersion.

Measurements were made of the change in the advancing face of the fluorescein sodium by using arbitrary intralenticular reference points. These included the end of the needle tract, small congenital defects, and/or a speck of debris which was carried in with the needle. Carbon granules placed on the outside of the penetrating needle are

deposited by the needle at intervals along the tract. These can be used for measurements at different positions. In these studies the magnification used was ×15.5, the slitbeam set at 0.2 mm. and the rheostat of the Bausch and Lomb Universal slitlamp kept at the midpoint position.

The border of the advancing fluorescein sodium is more difficult to define with increased magnification. At ×15.5 it has a fairly well-defined border but at ×31 power a faint greenish luminescence of much lower concentration, 0.05 to 0.1 mm. in depth, is seen to precede it. In order to obtain more reproducible results and measurements, the lower power of the slitlamp was used. The general topography of the intralenticular distribution was mapped in outline form using a slitbeam with the unaided eye and/or a loupe.

The rate of anterior cortical migration of fluorescein was determined in both eyes of two adults (three years and over) and two newly weaned (six weeks) animals. The average rate was found to be 1.3 mm. per 24 hr. in the adult and in the weaned. A probable error was introduced because of the difficulty in placing the slitlamp beam and microscope axis in the same angle on succeeding examinations. The rate of diffusion in the "clear" nuclear areas (infra) as contrasted with the cortical areas was definitely slower than the cortical movement and again approximately the same in both age groups. No figure is given for nuclear and posterior cortical movement as it was difficult to reproducibly measure with the instrument used.

*Weaned.* The general topographic picture is as follows: The fluorescein sodium spreads from the cortical area at the point of needle entrance both under the anterior and posterior capsules. The travel under the posterior capsule is completed by the second or third day when it can be seen advancing in the 6-o'clock area under the anterior capsule. The anterior cortex is stained and its margin can be seen under the anterior capsule in some animals as early as four hours postoperative. A slitlamp section through the original streak shows the pattern to be similar to a cross section of an umbrella, with the stalk of implanted fluorescein as the handle and the migrating material as the silk. Figure 3 shows the pattern of a slit section through the original deposit. The nuclear area even in this young age group may contain a small central core, one to two mm. in diameter, which remains clear long after it has been surrounded by fluorescein (fig. 4). The fluorescein sodium seems to diffuse generally without impedance throughout the rest of the nuclear area.

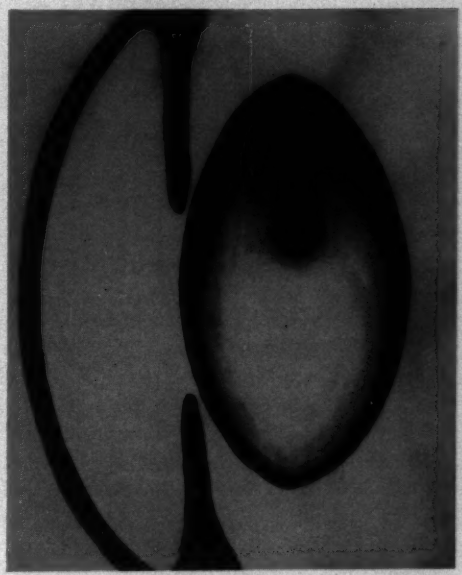

Fig. 3 (Vogel and Reinoehl). Umbrella-shaped cross section through fluorescein deposit shortly following implantation. Dye has migrated under both anterior and posterior capsules. Nuclear zone clearness is emphasized, and "porthole" is not included.

*Adult.* In the adult lens the cortical pattern of diffusion is essentially the same with spread both posteriorly and anteriorly. The central nuclear zone is clear much longer and as viewed grossly with the slit beam there is often formed a doughnut shaped fluorescence. The fluorescein sodium is much slower

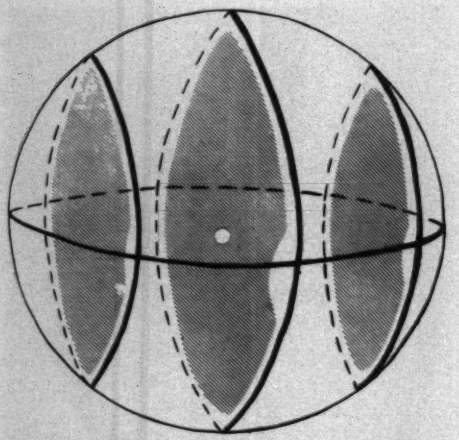

Fig. 4 (Vogel and Reinoehl). Note the small optically empty nuclear zone, the cleared cortical subcapsular region and the remains of the "port-hole" which has just about disappeared because of the clearing of the contrasting cortical fluorescein. (Intralenticular fluorescein sodium in vivo. Six-week-old rabbit; nine days after operation.)

in moving into the nuclear zone and this area is fluorescent long after the cortex is clear.

*Characteristics common to both age groups.* Fluorescein sodium follows the curve of the anterior capsule, as it diffuses through the cortex. No fluorescence of the capsule or the aqueous was seen in the areas overlying the stained cortex.

The fluorescein sodium is usually dense centrally and as it migrates it follows the capsule closely but does not penetrate it. The lens epithelium is apparently also stained by this heavy concentration. However, in several eyes in which the slit beam was moved into a less heavily stained area, it could be seen that the greatest clearing was in the immediate subcapsular region.

Nuclear retention of fluorescein sodium was seen in one eye of an adult animal for a period of eight months. Usually eyes were observed for a two to three-month period and, in all except one, nuclear fluorescence was seen until time of death. This indicates that fluorescein sodium is able to form a relatively insoluble bond with the constituents of the central lens area, as contrasted with the cortical areas, since the cortical areas usually cleared in two weeks (fig. 4).

The fluorescein migrating from above and below toward the central anterior subcapsular area, did not meet in the center of the lens. Instead, a central or slightly paracentral (inferiorly) clear zone about one mm. in depth and three to five mm. in diameter remained (fig. 4).

This is thought to indicate the presence of, either a region of relative impermeability or one in which there is swifter molecular movement due to higher permeability of the cortical substance in this area. This could result in undetectable fluorescence at any one time, giving what appears to be an optically empty "porthole." We are inclined to the latter viewpoint but studies will have to be made by placing fluorescein sodium directly into this area. The zone frequently was connected to the central nuclear clear zone in the three-year-old animals. This gave a somewhat dumb-bell shaped optically empty area surrounded by the fluorescence (fig. 5).

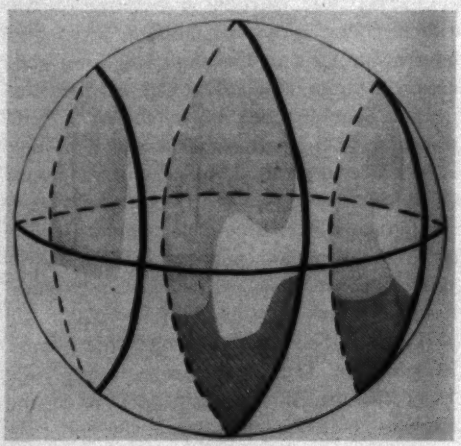

Fig. 5 (Vogel and Reinoehl). Cross section through lens nine days following intralenticular implantation of fluorescein sodium. Nuclear area and "port-hole" are connected forming a somewhat dumb-bell-shaped clear zone. Darker interior area is zone of lower concentration of fluorescein sodium. (Intralenticular fluorescein sodium in vivo. Three-year-old rabbit; nine days after operation.)

e. *Fluorescein sodium—alloxan-diabetic lens.* A fluorescein sodium pellet was implanted into the left eye of two alloxan-diabetic rabbits. The intralenticular implantation into the first animal (six weeks of age) was two weeks after giving 200 mg. of alloxan/kg. intravenously. Positive slitlamp examination at that time showed average height of silvery feathering of the posterior suture lines, to be 0.9 mm. Positive slitlamp examination of a second alloxan diabetic animal (six months of age) showed equatorial cortical vacuolization extending anteriorly to a clear central zone three mm. in diameter. The distribution of fluorescein sodium in these lenses was the same as that seen in the normal lens.

## II. Intralenticular fluid injections

The screening of a number of compounds for their usefulness in the pellet study shows that their number will be limited. If a pellet dissolves, the fibers surrounding it are subjected to a supersaturated solution which decreases in concentration the further out it diffuses from its origin. Stationary, sometimes double-walled or concentric heavy opacities developed within twenty-four hours around the following pellets: colchicine, sodium fluoride, potassium iodoacetate, a-naphthol and b-naphthol. The size varied from 0.3 to 0.5 mm. in diameter, and the surrounding lens matrix remained clear. It is possible that the toxic and/or denaturating effect of the overwhelming dose, to which the adjacent fibers were subjected, causes violent changes which disguise more subtle alterations which could be detected with lower concentrations. The following describes a technique of injecting minute quantities of fluid, intralenticularly, and observations on the one thus far used—methylene blue.

### A. Method

A capillary needle 50 μ diameter is drawn from standard 0.8 mm. bore capillary tubing and mounted in No. 60 polyethylene tubing (ID = 0.76 mm.).

The glass capillary is forced about two mm. into the polyethylene tubing. Heat is applied by a microflame until the polyethylene is soft. This is pinched tightly. On cooling there is enough constriction of the polyethylene to form a pressure bond with the glass. This system is attached to a Luer-lock tuberculin syringe placed into a modified frame of a standard micrometer. A scleral puncture hole is made with the pellet injector in the same location as used for the pellet implantation. The needle guard is set so that only the sclera is perforated (1.5 mm.). The same local anesthesia and positioning of the rabbit head and eye are used. Magnification is provided by a loupe and illumination is provided by a headlamp. The capillary needle is slowly introduced into the center of the lens (fig. 6). When sufficient pressure is built up in the capillary system to eject a small amount of fluid, the needle is withdrawn leaving a track similar to that seen in the fluorescein sodium pellet injection.

Methylene blue is frequently used in the study of cell respiration. It is a compound which retains color in the presence of oxygen or in the absence of reducing agents and is decolorized in the presence of a reducing

Fig. 6 (Vogel and Reinoehl). Micro-injection of the rabbit lens in vivo. Eye is proptosed and initial scleral puncture made with a No. 27 needle. A glass capillary needle 50 μ in diameter is inserted through the ciliary body into the lens. End of needle is guided under direct vision with a head light and loupe.

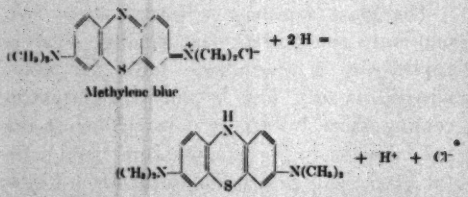


Fig. 7 (Vogel and Reinoehl). Structural formula of methylene blue before and after reduction with the hydrogen molecule. (From *Chemistry and Methods of Enzymes:* p. 202, Sumner and Somers.)

agent. If a suspension of cells is respiring in the absence of oxygen, the dye may act as a hydrogen acceptor, for the dehydrogenases of the cells. Acceptance of hydrogen changes it to the leuko-form (fig. 7). The amount of leuko-form produced can be used as measure of the dehydrogenase activity present. The substance which furnishes the hydrogen is the donator and the easily reduced substance is the acceptor. In this instance methylene blue is an artificial acceptor. Dehydrogenating enzymes usually exhibit marked specificity towards the donator but may be unspecific to the acceptor. Indophenol blue, thionine, dinitrobenzol, alizarin blue, plus other dyes, can be used in vitro as artificial acceptors.[5]

## B. Results

Work has been undertaken to study the respiration of the lens by the technique of intralenticular injection of minute amounts of methylene blue. The eye lends itself to this approach because of the clearness of its media. Figure 8 shows the appearance of a typical track of methylene blue extending from the equator of the lens through the cortex to the nuclear area. The cross hatched area shows the changing of the methylene blue to the leuko-form and the remainder is the as yet unreduced nuclear deposit.

Table 1 shows the time of disappearance of methylene blue as viewed through the slitlamp and/or the loupe in in vitro studies. The cortex for the purposes of this study was assumed to be the 1.5 mm. depth be-

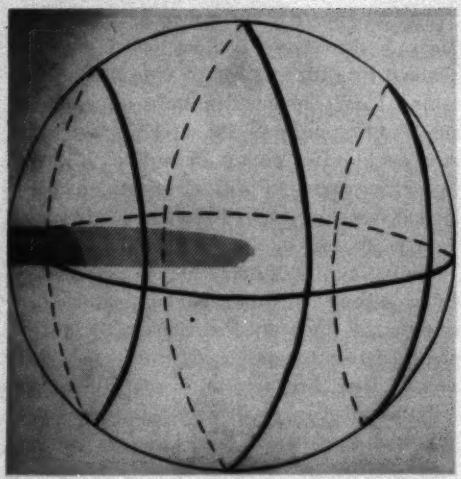

Fig. 8 (Vogel and Reinoehl). Diagram of methylene blue track extending from the cortex to the nucleus of the lens. Cross-hatched area represents the reduced form which has commenced in the cortical deposit.

neath the capsule.* Initial appearance of the leuko-compound in the cortical and nuclear areas was noted, along with the time needed for complete reduction of the volume of methylene blue deposited in those areas. These studies were carried out in vitro at 24°C. and 36°C. (under $N_2$ gas)† in moist chambers, and in vivo. The work reported is confined to the 15-day-old rabbit lens. The time necessary to detect the first cortical change to white, in vivo, was from five to 30 minutes. This broad time range can be accounted for by the difficulty in seeing the

* We selected this arbitrary depth of 1.5 mm. because of three incidental observations made in the course of this study: (a) fluorescein sodium seems to penetrate and clear from this area faster than from the rest of the lens, (b) internal trauma to the lens, as caused by an indwelling moving needle, will cause a globular central milky opacity which is clearly demarcated from the superficial 1.5 mm. of the cortex, (c) the early vacuoles of alloxan cataract seem to spare this region, forming in a clear line of "cleavage" about 1.0 to 1.5 mm. deep to the capsule.

† Studies at room temperature were not done under $N_2$. (It is recognized that this is a necessary step and will be reported later.)

TABLE 1

SHOWING IN VIVO AND VITRO REDUCTION OF METHYLENE BLUE AT ROOM TEMPERATURE AND 36°C. THE TIMES OF REDUCTION FOR THE IN VIVO AND VITRO AT 36°C. ARE APPROXIMATELY THE SAME. THAT FOR THE VITRO AT 24°C. NOTICABLY SLOWER

(Methylene blue 0.5-percent aqueous)

| | Time | | | | | |
|---|---|---|---|---|---|---|
| | Beginning Disappearance | | Complete | | | |
| Vivo 15 day old | Cortex | Nucleus | Cortex | Nucleus | | |
| 1183 O.S. | ? | ? | ? | 20 | | |
| 1183 O.D. | ? | 18 | 13 | 48 | | |
| 1186 O.S. | 5 | 10 | 10 | 31 | | |
| 1187 O.D. | 6 | 11 | 8 | 27 | | |
| 1187 O.S. | 6 | 14 | 14 | 22 | | |
| 1189 O.S. | 5 | 15 | 10 | 29 | | |
| 1190 O.D. | ? | 17 | 32 | 49 | | |
| 1190 O.S. | 30 | 54 | 54 | 69 | | |
| 36°C. N2 | | | | | Temp. Av. | |
| 1194 O.D. | 26 | 41 | 37 | 56 | 35° | |
| 1194 O.S. | 6 | 11 | 11 | 41 | 35° | |
| 1195 O.D. | 30(−) | 34 | 34 | 85 | 35.4° | refrig. 18 hr. prior to study |
| 1195 O.S. | Within 10 minutes | | | 18+ | | ant. capsule ruptured |
| 1196 O.D. | 6 | 16 | 6 | 29 | 37.4° | |
| 1196 O.S. | 16− | 16 | 16 | 29 | 35.5° | |
| 1197 O.D. | 15 | 20 | 23 | 29 | 36.5° | |
| 1197 O.S. | 13 | 26 | 26 | 35 | 36.5° | |
| Vitro Room Temp. 0ª 15 day old | | | | | | |
| 1190 O.D. | 28 | ? | 45 | ? | not in moist chamber; vitro is *2nd* inj. | |
| 1190 O.S. | ? | ? | ? | 195 | | |
| 1191 O.D. | 47 | 109 | 109 | 192+ | | |
| 1192 O.D. | 42 | 210 | 125 | 280 | | |
| 1192 O.S. | 45 | 120 | 79 | 410 | | |
| Average 24°C. | 38 | 146 | 89 | 269 | | |
| Average 36°C.; N2 | 16 | 23 | 22 | 40 | | |
| Average Vivo | 10 | 20 | 20 | 38 | | |

extreme equatorial deposit and the variation in amount of methylene blue deposited. The average time required for complete reduction of the acceptor in the cortex was twenty minutes. The nuclear areas were noticeably slower in their reduction. It is apparent that the largest amount of methylene blue is in the nuclear area, and that this might account for the time lag. Preliminary quantitative studies were made by plotting $k = \frac{1}{t} \ln \frac{a}{a-x}$ and using $\ln \frac{a}{a-x}$ as ordinate and t as abcissa.* The slope of the graph for the cortical region is steeper than the slope of the straight line representing the reduction of the material in the nuclear region. This indicates that the rate of reduction is greater in the more superficial 1.5 mm. layer.

The times of reduction in vitro at an average temperature of 36°C. are close to those in vivo. At a temperature of 24°C. it can be seen that there is a marked slowing of the reduction of the methylene blue, particularly in the nuclear area. It is well known that enzyme systems are very sensitive to temperature. A rough rule of thumb by Van Hoff states that a rise of 10°C. will double the speed of a chemical reaction. For

* a = original volume of methylene blue; x = reduced methylene blue; t = time in minutes; k = coefficient of reduction; ln = natural log.

the cortical region in this study the temperature coefficient is roughly four and for the nuclear region 10 per 12°C. This suggests that there are probably factors other than enzymatic causing this. One possibility is that in vivo, in contrast to an in vitro system such as a homogenate, it may be necessary for the acceptor to come into a more precise spatial contact with the donator system, that is, it must diffuse through the cell membranes in order to be reduced, and/or the donator electron must move towards the methylene blue. This movement may be hindered by lower temperature.


## Summary

Two methods of in vivo and in vitro lens studies have been described. The Kaczurowski pellet injector deposits discrete entities of substances intralenticularly, and a microinjector can be used to inject fluids of various concentrations. Studies thus far have indicated the injection of fluids to be the more feasible method. Preliminary work showing the reduction of methylene blue by the lens in vivo and vitro, and diffusion studies of fluorescein sodium in vivo are presented.


*1601 Spring Garden Street (30).*

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## Discussion

Dr. Ludwig von Sallmann (Bethesda, Maryland): Dr. Vogel has introduced a new technical approach for studies of physico-chemical properties of the crystalline lens or components of the lens and possibly for studies of enzyme systems of the living lens. This technique, as far as I know, is completely new, and therefore whatever I say in discussion is not more than speculation, and should be taken as such.

Injection of microvolumes of a test substance, either in form of particulate matter or solutions is reminiscent of the experiments of biologists who subject animal cells as arbacia and starfish eggs or plant cells to similar procedures and obtain valuable information for instance, on the rate of penetration of hydrogen and hydroxylions, the diffusion of weak and strong bases and acids and allied biological observations.

In such a complex organ as the lens with its zones of discontinuity, adsorption phenomena and effects of relative frictional resistance also might possibly be established by Dr. Vogel's technique and open avenues of which little is known indeed. Slitlamp observations appear well suited to localize such events on boundaries and interphases.

It might be too early at this stage of the pioneering work of Dr. Vogel to ask him whether the dependence of the diffusion rate on the dissociation constant of the injected material could be shown and—this is the more essential question—to which extent the artificial stretching and disorganization of fiber elements by the injection pressure alter the situation as it may exist in the intact lens in vivo.

I ask this question in view of initial observations encountered in our attempts to inject a fixative into the lens. On request of Dr. Wanko a small quantity of osmium tetroxide was introduced in the living rabbit lens through a 30-gauge stainless steel needle in order to prepare the lens for electron microscopic studies of the fibers. On dissection of these eyes it was clear that the injection pressure had caused a rupture of the lens capsule. I feel now that the elegant technique of Dr. Vogel would have provided a better way to accomplish the task.

Dr. Adolph W. Vogel (closing): Thank you, Dr. von Sallmann. I am not prepared to answer your first question. However, as far as the disturbance of the lens is concerned, we turned to the capillary needle because, as we use it, it is actually about one-fifth the diameter of a No. 32 needle, the least traumatic instrument with which we started.

I have not experienced stretching of the lens because I have not used a fixative as an injection material. I wonder if perhaps the fixative which was used formed a plug around the shaft of the needle and thereby prevented regurgitation of any fluid along the shaft of the needle. We have found that the methylene blue will occasionally be deposited outside in the zonular fibers.

# IN VITRO LENS STUDIES

## II. Production of Cataractous Changes*

Marguerite A. Constant, Ph.D.
*Saint Louis, Missouri*

### Introduction

Cataractous changes have been produced in vivo in experimental animals by a variety of methods including radiomimetric agents, diabetes-producing compounds, nutritional disturbances and toxic compounds such as naphthalene, and so forth.[1,2] Lenses have been made opaque and cataractous in vitro by inhibitors of glycolytic or respiratory metabolic pathways.[3] Recently von Sallmann has reported[4] the interesting observation that several cataractogenic agents such as Myleran®† (1,4 dimethanesulfonoxybutane), galactose, alloxan and irradiation alter the mitotic activity of the lens epithelium of rats in vivo.

In preliminary in vitro lens culture experiments in this laboratory,[5] Myleran or alloxan did not significantly alter the mitotic activity of rabbit lenses cultured for 12 hours. However, during the course of these studies it was found that methylene blue caused an inhibition in mitosis of the lens epithelium. Furthermore, vacuolar changes in the anterior portion of the lens were noted in a 12-hour culture period. It is the purpose of the present report to present the preliminary findings of the effect of methylene blue and other compounds on lenses in vitro.

### Experimental

Lenses were cultured by the technique previously described.[5] By use of this procedure the mitotic activity of lens epithelium could be successfully maintained for 16 hours. The media used was TC199 (Difco) diluted by nine to 29 percent with 1.2-percent hypertonic tris hydroxymethylaminomethane, pH 7.5. Lenses were cultured for periods of four to 12 hours.

The compounds studied were methylene blue (several sources), thionin, toluidine blue, Evans blue, indigo disulfonate, indigo tetrasulfonate, 2,6 dichlorophenolindophenol, 2,4 dinitrophenol, and triphenyltetrazolium chloride. These compounds were dissolved in sterile saline at a concentration of 50 mg. percent and 0.1 or 0.2 cc. was added to the culture tube containing 5.0 cc. of media. The concentration of these compounds in the media was approximately $10^{-5}$ M.

At the end of the experiment each lens was observed for clarity and gross (color) changes. In most cases the lenses were so clear that posterior or anterior vacuolar changes could not easily be seen with the naked eye. Each lens was put in a shallow dish containing the media in which it was cultured (or in similar media) and examined with the aid of a microscope (×20 to ×60 magnification). Photomicrographs of these vacuoles were taken by focusing on the anterior surface of the lens; thus Figures 1 and 2 depict the topography of such lenses. Flat preparations of lens epithelium for mitotic counts were made following the method of Howard as modified by von Sallmann.[6] Histologic sections of lenses were made in the usual manner and stained with hematoxylin and eosin.

### Results

#### Mitotic Activity of Epithelium

Data on the effect of these compounds on the mitotic activity of lens epithelium are

* From the Department of Ophthalmology and the Oscar Johnson Institute, Washington University School of Medicine, Saint Louis. This research was supported in part by the USAF under Contract No. AF 18 (600)-1269; monitored by the USAF School of Aviation Medicine, Randolph Field, Texas.

† Myleran supplied through the courtesy of the Burroughs Wellcome and Company, Inc., Tuckahoe, New York.

tabulated in Table 1. In the presence of methylene blue at a concentration of $2 \times 10^{-5}$ M mitosis was almost completely inhibited at four hours and remained suppressed for 12 hours. Toluidine blue caused a similar marked decrease in mitotic activity. Although mitosis was inhibited in the presence of triphenyltetrazolium, Evans blue, thionin or 2,4 dinitrophenol, the degree of inhibition was not as great as that caused by methylene blue. Mitotic activity was normal in the epithelium of lenses cultured in media containing 2,6 dichlorophenolindophenol or indigo disulfonate and was slightly decreased when the media contained indigo tetrasulfonate.

### Vacuolar changes

With the exception of Evans blue and indigo di- or tetrasulfonate which were not

TABLE 1

Effect of various substances on the mitotic activity of epithelium and vacuole formation of cultured lenses

| Substance | Conc. in Media (molar) | No. of Lenses | Culture Time (hr.) | Average No. of Mitotic Cells/Epithelium | Severity of Vacuole Formation‡ |
|---|---|---|---|---|---|
| — | — | 5 | 8 | 247 | 0 to 1 |
| Methylene blue | $5\times10^{-5}$ | 5 | 8 | 17 | 2 to 4 |
| — | — | 3* | 6 | 290 | 0 to 1 |
| Methylene blue | $2\times10^{-5}$ | 3* | 6 | 0 | 2 to 4 |
| Methylene blue | $2\times10^{-5}$ | 2 | 4 | 5 | 2 |
| Methylene blue | $2\times10^{-5}$ | 2 | 8 | 0 | 3 |
| Methylene blue | $2\times10^{-5}$ | 2 | 12 | 1 | 2 to 4 |
| Methylene blue and ascorbic acid† | $2\times10^{-5}$ | 2 | 12 | 0 | 3 to 5 |
| Toluidine blue | $6\times10^{-5}$ | 3 | 12 | 20§ | 2 to 3 |
| Methylene blue | $5\times10$ | 3 | 12 | 3 | 2 to 4 |
| — | — | 7 | 8 | 201 | 0 to 1 |
| Triphenyltetrazolium | $1\times10^{-4}$ | 7 | 8 | 34 | 2 to 3 |
| Evans blue | $2\times10^{-5}$ | 3 | 12 | 51 | 0 to 1 |
| Methylene blue | $5\times10^{-5}$ | 3 | 12 | 4 | 2 to 4 |
| — | — | 3 | 12 | 177 | 0 to 1 |
| Thionin | $7\times10^{-5}$ | 3 | 12 | 46 | 2 to 5 |
| Thionin | $7\times10^{-5}$ | 2 | 12 | 52 | 2 to 4 |
| Thionin + ascorbic acid† | $7\times10^{-5}$ | 2 | 12 | 25 | 3 to 5 |
| 2, 4 dinitrophenol | $?\times10^{-5}$ | 2 | 12 | 72 | 2 to 3 |
| Thionin | $7\times10^{-5}$ | 2 | 12 | 72 | 2 to 4 |
| — | — | 3 | 12 | 107 | 0 to 1 |
| 2, 6 dichlorophenolindophenol | $7\times10^{-5}$ | 3 | 12 | 145 | 4 to 5 |
| — | — | 2 | 12 | 259 | 0 to 1 |
| Indigo disulfonate | $4\times10^{-5}$ | 2 | 12 | 241 | 0 to 1 |
| — | — | 3 | 12 | 138 | 0 to 1 |
| Indigo tetrasulfonate | $4\times10^{-5}$ | 3 | 12 | 93 | 0 to 1 |

* Unbuffered media.

† 20 mg. percent, added as ascorbic acid or calcium ascorbate.

‡ Evaluated from 0 to 5. Examples of changes evaluated at 5, 3, and 2 are shown in Figures 1D and 1E respectively, in instances of lenses cultured with thionin. The difference in size and numbers of vacuoles and surface area involved made the quantitative comparison of the effects of these compounds difficult.

§ One preparation showed numerous metaphases.

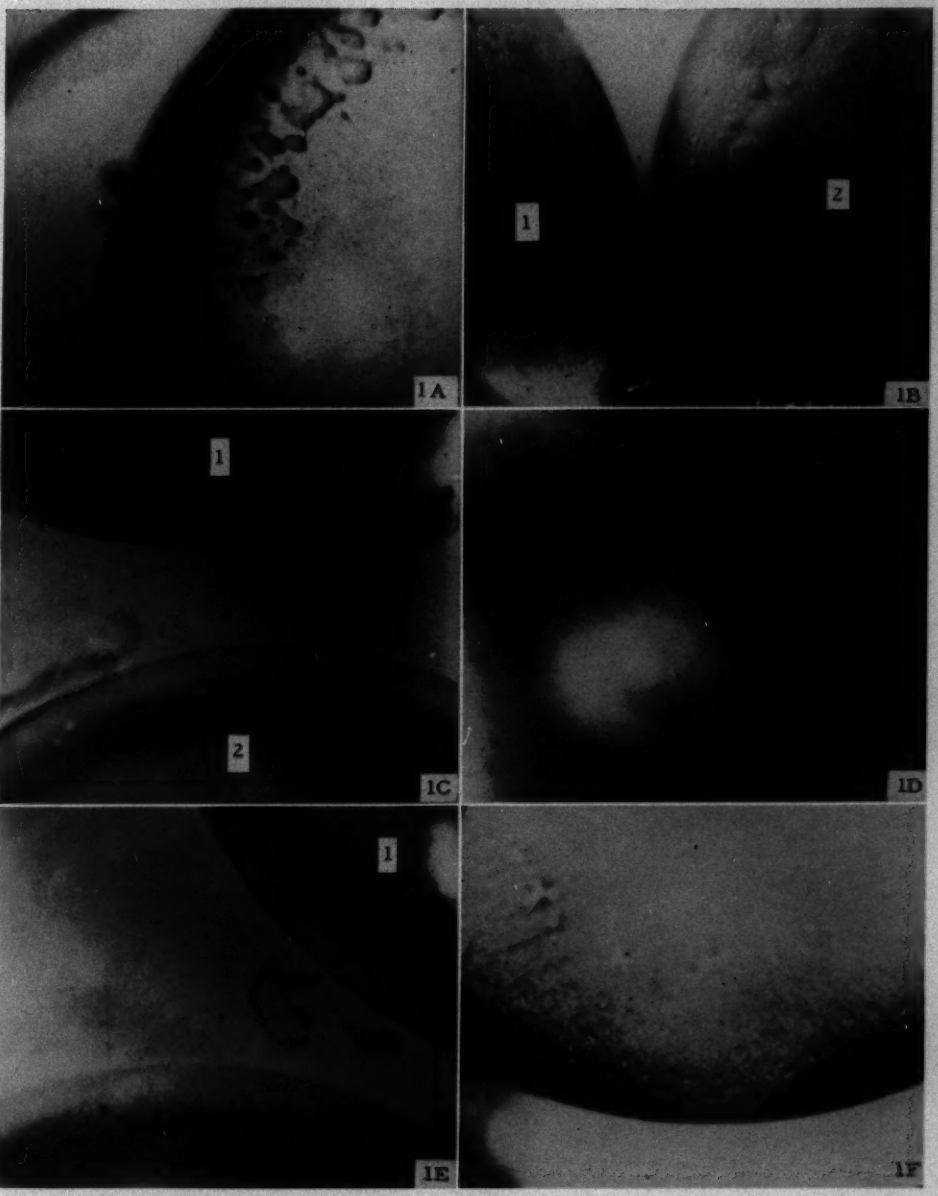


Fig. 1 (Constant). Photomicrographs of the topographic appearance and distribution of vacuoles in the anterior portion of the lens.

(1A) Severe vacuole formation caused by methylene blue.

(1B) Increased severity of cataractous changes caused by ascorbate (lens 2) compared to the control, contralateral lens (lens 1); both lenses cultured in the presence of methylene blue. The lenses in Figure 1A and Figure 1B were all deeply stained.

(1C) An instance of marked resistance of lenses to cataractous changes. Lens 1 was cultured with methylene blue and the contralateral lens (2) with methylene blue plus ascorbate. These lenses were only slightly stained.

(1D) Severe vacuolar changes caused by thionin.

(1E) Increased severity of cataractous changes caused by ascorbate (lens 2) compared to the contralateral lens (lens 1); both lenses cultured with thionin.

(1F) Typical appearance of vacuoles caused by toluidine blue. These lenses are usually deeply stained.

found to be cataractogenic, the compounds studied caused vacuole formation which appeared to be fairly characteristic in size and distribution for a given compound. These vacuolar changes occurred in the peripheral one fifth to one half of the anterior surface of the lens and generally were uniformly distributed over the circumference with the pattern in one quadrant similar to that of the next, and so forth. An exception to this uniform distribution was apparent in the following instances:

A. When the media contained triphenyltetrazolium or 2,4 dinitrophenol.

B. When bulging of lens substance occurred.

C. When a fairly large portion of ciliary body (2-3 mm.) was adherent to the lens.

In the case of A, only one quadrant might be involved with the vacuoles extending almost to the center of the lens. When B and C occurred, the area adjacent generally showed greater involvement than other quadrants. When viewed with the slitlamp, these vacuoles appeared to be subcapsular.

An instance of severe vacuolar changes caused by methylene blue is shown in Figure 1A which is a photomicrograph of the topographical appearance of the anterior peripheral surface of the lens. The predominant vacuoles are large, slightly irregular in shape with sharply delineated edges. Blister formation with very indistinct edges gradually sloping into the contour of the lens may be produced by taking a cultured lens from media and putting it into saline. As indicated, these blisters are quite distinct from the vacuoles formed during culture. It was found that the addition of ascorbate to the media increased the severity of the vacuolar changes (fig. 1B). In both of these experiments the epithelium (especially the peripheral one fourth) was deeply stained. However, in other experiments in which the epithelium was slightly stained results similar to that shown in Figure 1B were obtained. It would appear that although the staining of cells may be associated with the toxicity of methylene blue, the two findings are not directly proportional. A similar lack of correlation between staining and toxicity has been observed in four- and eight-hour studies with methylene blue or thionin and 12-hour cultures with 2,6 dichlorophenolindophenol.

Thionin caused the formation of regularly dispersed medium to small vacuoles at the very periphery of the anterior surface (fig. 1D). The presence of additional ascorbic acid in the media also aggravated the cataractous process in these lenses (fig. 1E). In 12-hour cultures with toluidine blue, large vacuoles predominated with characteristic staining of clumps of cells near the center of the vacuoles (fig. 1F). However, recent studies with four- and eight-hour cultures showed relatively little staining of the lens at four hours and it was observed that the staining of the epithelium in longer cultures hid the presence of numerous smaller vacuoles at the periphery. The severity of this vacuolar change was approximately twice as great at 8 hours and only slightly more severe at 12 hours.

Lenses cultured in media containing 2,6 dichlorophenolindophenol were not stained by the dye but both the posterior and anterior surface of the lens was cloudy. This cloudiness was in contrast to the effect of the other cataractogenic compounds which when viewed from the posterior surface to avoid interference from staining, or in instances of very faint staining, the lens was found to be clear. Indophenol caused the most consistently severe vacuolar changes with various-sized vacuoles at the periphery and medium to very large vacuoles extending almost to the center of the lens (fig. 2A).

Less severe vacuolar changes were caused by tetrazolium or 2,4 dinitrophenol. In the case of dinitrophenol, the concentration in the media was not as high as with the other compounds because of its low solubility. The vacuoles caused by tetrazolium were not necessarily associated with the points of maximum formation of the formazan (fig.

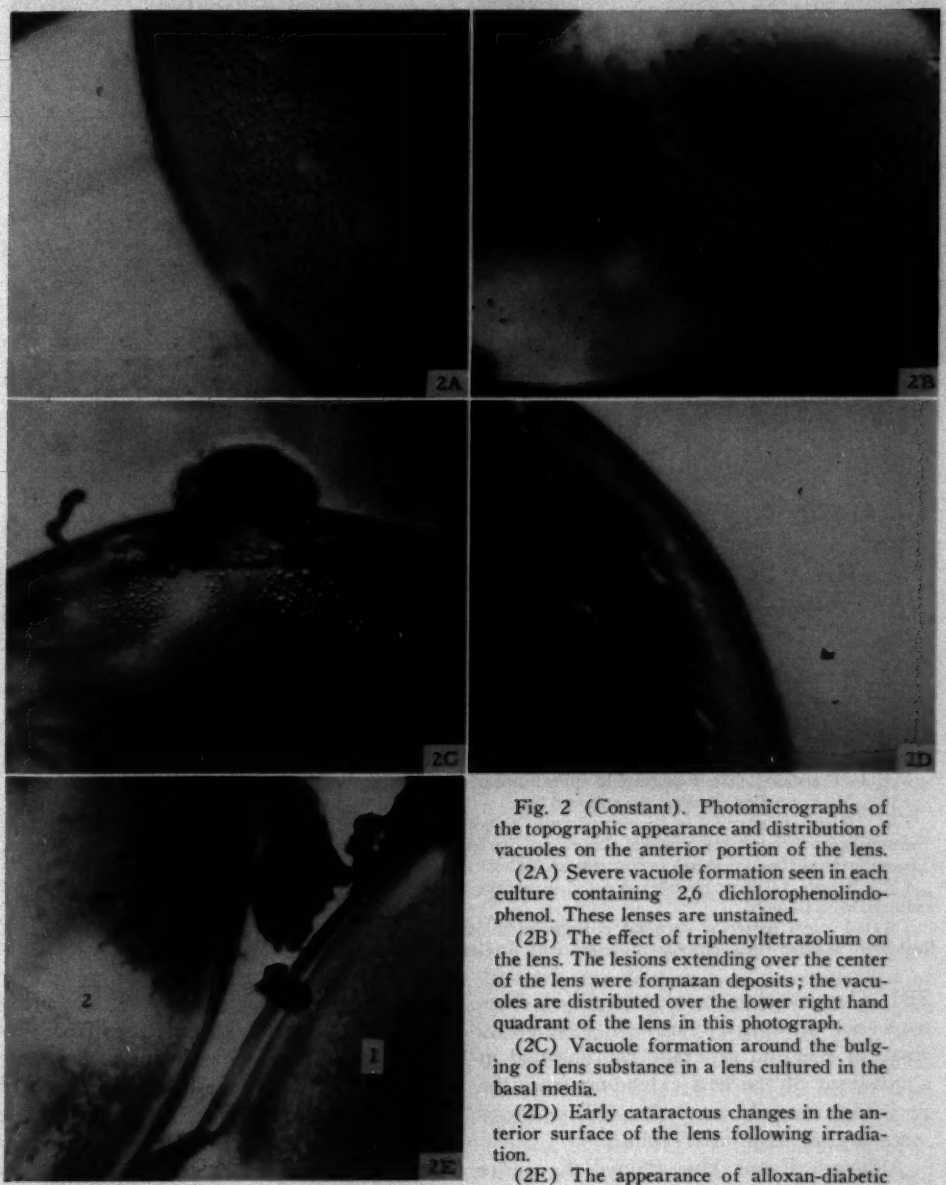


Fig. 2 (Constant). Photomicrographs of the topographic appearance and distribution of vacuoles on the anterior portion of the lens.

(2A) Severe vacuole formation seen in each culture containing 2,6 dichlorophenolindophenol. These lenses are unstained.

(2B) The effect of triphenyltetrazolium on the lens. The lesions extending over the center of the lens were formazan deposits; the vacuoles are distributed over the lower right hand quadrant of the lens in this photograph.

(2C) Vacuole formation around the bulging of lens substance in a lens cultured in the basal media.

(2D) Early cataractous changes in the anterior surface of the lens following irradiation.

(2E) The appearance of alloxan-diabetic vacuolar changes (lens 2) photographed by a similar means. Lens 1 is a control uncultured lens.

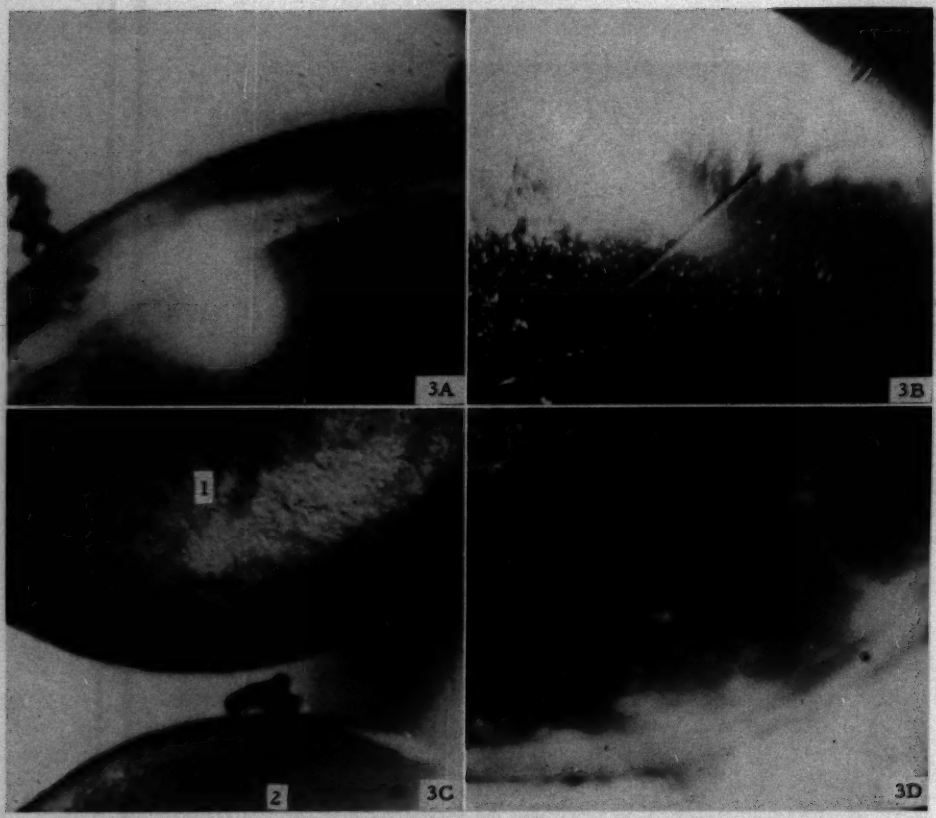


Fig. 3 (Constant). The topographic appearance of vacuoles at the posterior surface of the lens.
(3A) Mild vacuolar changes in a cultured lens.
(3B) Moderate changes in a cultured lens. A hair put under the dish containing the lens in media was focused upon through the anterior portion of the lens to demonstrate the clarity of these affected lenses. The anterior surface of lenses in Figures 3A and 3B showed no changes.
(3C) Severe vacuolar changes. Lens 2 shows the anterior portion of the contralateral lens; both lenses cultured with thionin.
(3D) Typical posterior vacuolar changes in an irradiated lens with peripheral large vacuolar and central fine "feathery" vacuolar involvement with a relatively clear intermediate zone.

2B). The vacuolar changes caused by dinitrophenol or tetrazolium were medium to small in size and generally concentrated in a quadrant of the lens extending from the periphery towards the center. These lenses were crystal clear after culture.

Although Evans blue stained the entire capsule, no vacuolar changes were observed in these lenses. Similarly negative results were obtained with indigo di- and tetrasulfonate which did not stain the lens (fig. 2).

The cataractogenicity of these compounds was found to vary with the lenses from different individuals. Thus in instances where the effect of methylene blue was compared with that of thionin on the lenses of the same rabbit, if one dye produced severe vacuolar changes, the other was also comparatively severely cataractogenic; similarly if one lens showed only mild to moderate changes, the contralateral lens was also relatively resistant to the action of these compounds. This ap-

parently was not due to unknown differences in the media or dyes from one culture to another as contrasting effects of the same dye on the lenses of two different animals was apparent in the same experiment (figs. 1A through 1C).

As the number of cultured lenses increased to approximately 150 with approximately 30 percent of these being control contralateral lenses cultured in the basal media, it became apparent that minor vacuolar changes were frequently seen in these control lenses. The vacuoles were very small and in the majority of cases very few in number. These vacuoles were insignificant compared to those caused by the various compounds used in these studies. There was no inhibition of mitosis, cytopathology in flat preparations or histopathology in microscopic sections of these lenses. All criteria used in previous lens culture studies have indicated that the culture procedure maintained a normal lens for 16 hours. The occurrence of these vacuoles seen only with the aid of a microscope in an otherwise normal lens raises the question as to the validity of studies of the chemistry of the "normal" cultured lens. However, a concentration of larger vacuoles was seen in almost all instances where a break in the capsule occurred during culture (fig. 2D). Such weakening of the capsule was not necessary to initiate the cataractous changes and in 90 percent of our cultured lenses the capsule was intact.

In almost every instance, if one observed the lenses under the microscope, small vacuoles were found on the posterior facet of the lens. In approximately 25 percent of the cases, this involvement was very minor (fig. 3D). It is difficult to evaluate the severity of the posterior vacuolar changes in relation to the changes on the anterior surface of the lens due to the difficulty in quantitating these changes. However, instances of moderate posterior changes were seen in the absence of anterior involvement and it would seem that the two processes are not directly related. The posterior vacuoles are presumably due to contact with the glass of the culture tube or may be initiated by slight adherence of the capsule to the polyethylene loop used in transferring the lenses to the culture tube.

### Histopathology

In all instances of methylene blue or tetrazolium experiments, examination of the flat preparations, which were stained with nuclear stains only (Feulgen or toluidine blue), showed the presence of pathologic changes in the epithelium. These compounds in the media caused the formation of giant, pale nuclei most frequently seen in the germinative zone (fig. 4A). Such degenerative cells were seen in small numbers (approximately 50/epithelium) at four hours and were present in large numbers (especially methylene blue cultures) at eight hours. By 12 hours, and frequently in earlier cultures, methylene blue caused the formation of lesions of the epithelium consisting of several cells with giant nuclei and frequently pyknotic nuclei (fig. 4A). The lesions caused by tetrazolium were not seen to progress beyond the isolated giant nuclei stage during the culture periods of the present studies. The presence of additional ascorbic acid in the media aggravated this pathologic change (figs. 4B and 4C). Epithelium of lenses cultured in 2,4 dinitrophenol frequently contained some pyknotic nuclei. The flat preparations of epithelium in other instances were not remarkable.

The histologic sections of lenses cultured in methylene blue showed accumulations of epithelial cells in lesions corresponding to that seen in the flat preparations (fig. 5A). A section through the central vacuolar lesions of a lens cultured in media with methylene blue and ascorbic acid is shown in Figures 5C and 5D. Cortical involvement in this process was insignificant. Fairly marked vacuolar changes were seen in one lens cultured in thionin (fig. 5B). In general, thionin and toluidine blue caused more vacuolar changes in the cortex than did methyl-

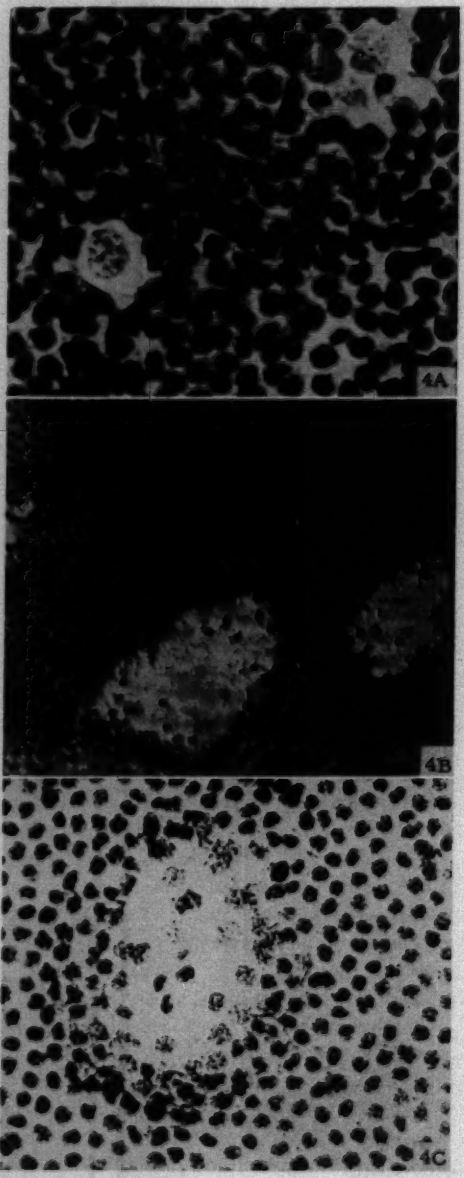

Fig. 4 (Constant). Pathologic changes observed in flat preparations of lenses cultured in media containing methylene blue or triphenyltetrazolium.

(4A) Large pale nuclei seen in both instances. Groups of these cells (upper right hand corner) were seen only in cases of methylene blue cultures.

(4B) Large lesions seen in epithelium of lenses cultured in methylene blue plus ascorbate.

(4C) Higher magnification of such lesions which consist of the cells with giant nuclei and pyknotic cells.

ene blue but the extent of this involvement was not very great in the sections of lenses studied to date. In one lens cultured in 2,6 dichloroindophenol, a subepithelial fine line of basophilic material was seen; this material was also present between cells in the epithelium. In general, however, with the exception of lenses cultured in methylene blue containing media, the severe vacuolar changes seen grossly were not reflected by pathologic changes microscopically in these lenses cultured for 12 hours. It is possible that since serial sections were not done that the sections may not represent areas of maximum involvement.

In a preliminary experiment it was found that lenses cultured in media containing thionin and additional cysteine showed areas of accumulation of epithelial cells and in one instance a nest of cells posterior to the bow (fig. 5F). It would seem possible to alter the pathologic effects of these dyes on the lens and further work along this line is underway.

## Discussion

One of the challenging problems in the study of the pathogenesis of cataract is the elucidation of the early processes occurring prior to the development of opacities. Recent in vivo studies by von Sallmann[4] have shown an effect of several cataractogenic agents on the mitotic activity of lens epithelium. Some agents caused an inhibition of mitosis for the first few days followed by an increase in mitotic activity, whereas others caused an increase in number of mitotic cells in lens epithelium from the onset of their administration. A second divergent action of metabolic states or agents causing cataracts was that in some instances cataract development was associated primarily with lens fiber changes and in other cases lesions of the germinative zone of the epithelium was the

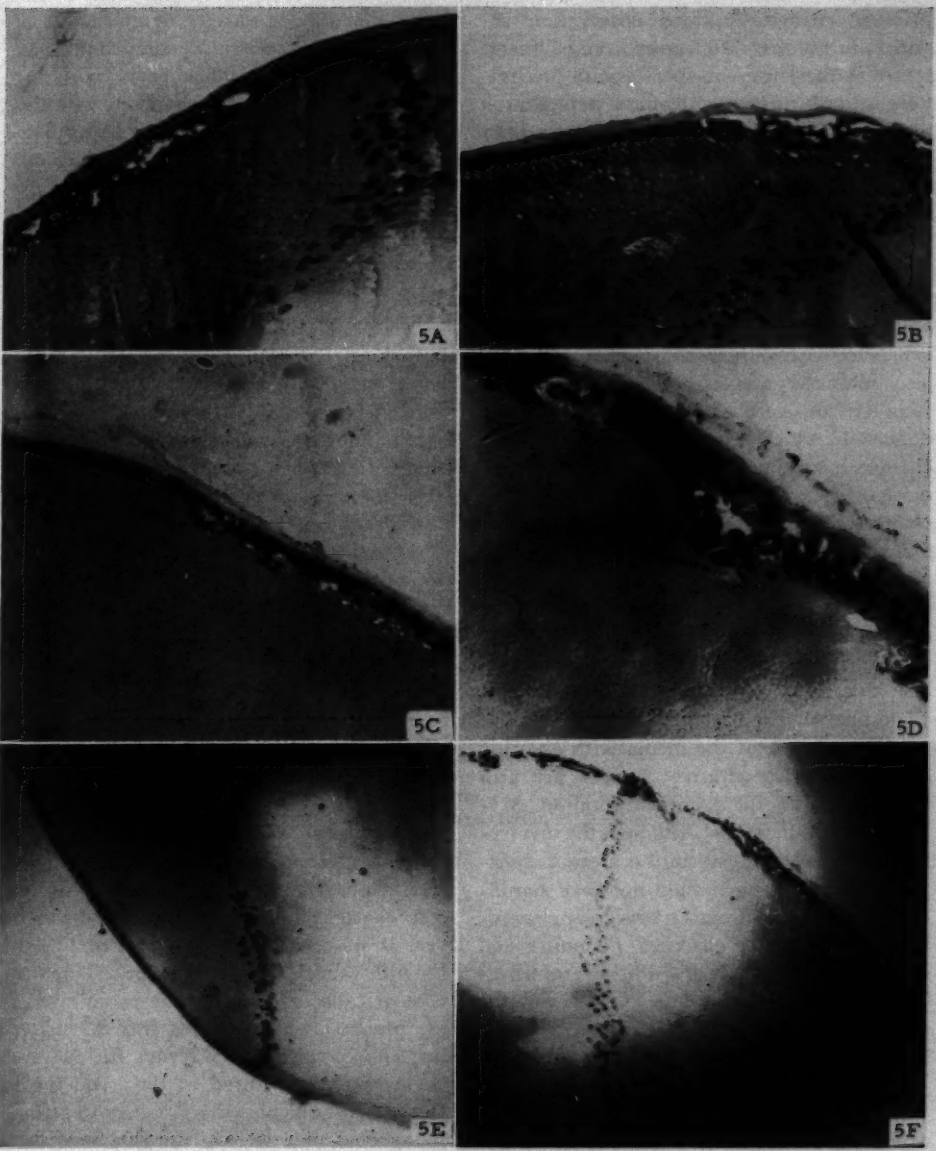


Fig. 5 (Constant). Histologic sections of cultured lenses.
(5A) Methylene blue-marked cell proliferation near the equator.
(5B) Thionin-marked vacuolar changes in the lens cortex.
(5C) Methylene blue and ascorbate section through vacuoles occurring centrally.
(5D) Higher magnification of the lesions seen in Figure 5C.
(5E) Indigo disulfonate. No changes observed microscopically.
(5F) Thionin plus cysteine proliferation of cells near equator. A nest of cells is seen posterior to the equator.

primary change. A third observation reported in the vivo studies by von Sallmann was that the lenses of species with low mitotic activity in lens epithelium were slower to develop opacities after irradiation than lenses with a high mitotic index.

Although many objections have been raised to the use of toxic substances such as the dyes and chemicals used in the present studies[7] there appeared to be several observations in these short-term culture studies which parallel findings in vivo. Most of the substances which caused vacuolar changes also inhibited mitosis. An exception was found in the case of 2,6 dichlorophenolindophenol which caused severe vacuolar changes but did not inhibit mitosis. Although most of these agents which caused vacuolar changes were without marked effect on epithelial cells two compounds (methylene blue and triphenyltetrazolium) caused degenerative changes in the form of giant pale nuclei in both instances and progressive degeneration with pyknotic cells in the case of methylene blue. This toxic manifestation of methylene blue was consistently increased by the supplementation of the media with ascorbic acid. These degenerative changes are also seen following the administration of Myleran[4] or irradiation.[4,8] Although the involvement of the lens fibers in the present short-term studies was generally not very significant, an instance of marked vacuolar changes in lens fibers was observed (thionin) and mild changes were observed in several lenses (thionin and toluidine blue). However, the severity of the vacuole formation seen grossly did not appear to be reflected in the microscopic sections studied. A variance in the susceptibility of lenses cultured in vitro from different animals to the development of cataractous changes was observed. Similar susceptibility differences are commonly seen in in vivo studies. These results indicate that the culture technique may prove useful in studying the factors related to the susceptibility of the lens to cataract development.

The dyes and chemicals used in the present studies have a variety of effects on organisms and a common denominator of action is not apparent. The basic dyes have been found to inhibit several enzyme systems[9,10] and to increase corneal hydration.[11] However, acidic dyes, including 2,6 dichlorophenolindophenol,[11] were not toxic in contrast to the findings in the present study.

In the limited series of substances studied in these experiments, a feature which appears to be common to compounds causing vacuoles is a positive $E_o$ potential. Determinations of the potential of the media before and after culture were made but the results were inconclusive. Unfortunately, these determinations were made after samples had been withdrawn for other purposes and possible changes occurring during these manipulations could not be excluded. In a few experiments it appeared that the potential of the media was slightly lower immediately after culture (approximately 50 mv. less) than the initial average value (+125 mv.) but in other instances measured after several manipulations had been made was found to be higher. Whether these changes are related to the effect of the culture or to technical difficulties cannot be ascertained at this time.

It is planned to expand the present series of experiments to clarify the possible relationship of oxidation-reduction potential to cataractous changes. Determination of glucose concentration in the media did not reveal an association of inhibition of glycolysis with vacuole formation. The average uptake of glucose by seven lenses cultured in the presence of basic dyes was 3.9 mg./24 hr./lens compared to 3.7 mg. for the contralateral lens cultured in the basal media. In two experiments with 2,6 dichlorophenolindophenol, the decrease in glucose content of the media was equivalent to an uptake of 3.4 mg./24 hr. by the lens compared to 3.2 mg. for the contralateral control lens. Similar results were obtained in experiments with the indigo dyes which did not cause vacuoles.

In view of the pathologic changes in the lens and epithelium caused by various agents

used in the present study, their counterpart in vivo, and the fact that this pathology apparently may be altered by appropriate changes in the media, it is felt that this culture technique may be of value in elucidating the early changes occurring during cataract velopment.

## Summary

The production by various agents of vacuolar changes in the cultured lens was studied. Compounds which caused vacuole formation were methylene blue, toluidine blue, thionin, triphenyltetrazolium chloride, 2,4 dinitrophenol and 2,6 dichlorophenolindophenol. Negative results were obtained with Evans blue, indigo di- and tetrasulfonate.

With the exception of 2,6 dichlorophenolindophenol, the cataractogenic agents inhibited the mitotic activity of lens epithelium. Evans blue, which appeared noncataractogenic, also seemed to be a mitotic inhibitor

In general the severity of the vacuole formation seen grossly was not reflected in histopatholic changes. However, in the presence of methylene blue (and tetrazolium) degenerative changes in lens epithelium occurred which resembled the pathology of cataractous changes produced in vivo. Preliminary studies indicated that the in vitro histopathologic manifestations of some of these agents may be altered by appropriate changes in the culture media.

*640 South Kingshighway (10).*

### Acknowledgments

I wish to express my gratitude to Dr. Bernard Becker for his guidance and suggestions throughout these studies, and to Miss Rosalie Smith for her technical assistance.

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## Discussion

Dr. Carl Wachtl (Detroit): Dr. Constant in her interesting studies has shown that vacuolar changes which often precede cataract formation can be produced in short-term cultures. The causative agents employed belonged to a variety of compounds. Among them was a dye, Evans blue, an oxidizing agent, 2,6 dichlorophenolindophenol and an uncoupler of phosphorylation, 2,4 dinitrophenol. Some of the substances employed also caused reduced mitosis. However, it was demonstrated that the two events, vacuolization and inhibition of mitosis, were not necessarily connected with one another. As an example, 2,6 dichlorophenolindophenol brought about a very severe incidence of vacuole formation and did not inhibit mitosis while Evans blue did not cause vacuole formation, but inhibited mitosis to some extent. Since it seems likely that vacuolar changes and mitosis are mediated by different mechanisms, the use of many different compounds gave rise to results of a variegated nature. These findings are difficult to interpret in view of our lack of knowledge concerning the specific mechanisms involved.

Dr. von Sallmann in his extensive and excellent

studies has shown that both a change in the mitotic rate, either a decrease or an increase, and vacuolization can lead to irreversible changes. However, if the germinative zone of the lens epithelium is only slightly affected, with the exception of its mitotic activity, these changes can be reversed upon withdrawal of the injurious agent. This, for instance, is true in galactose and alloxan cataracts. It might, perhaps, be interesting to know whether the effects of some of the agents employed by Dr. Constant are reversible.

At this point, I might mention that, although cataracts have been produced in vitro by metabolic poisons, experiments involving slower acting agents have seldom been attempted because of the problems encountered when lenses are cultured for long periods of time. One such attempt was Bakker's perfusion study in 1939. He observed minute opacities in explanted rat lenses which had been cultured for nine days in a medium containing 600 mg. percent of galactose. The sizes of these opacities, however, were insignificant compared with those occurring in vivo.

Dr. Constant intends to study more directly a possible relationship between oxidation-reduction potential and cataractous changes. This seems to be a good idea.

I wonder whether Dr. Constant has any theory or cares to speculate as to the possible reason why added ascorbate (20 mg. percent) increased the severity of the effects of methylene blue and thionin.

I would like to take this opportunity to commend Dr. Constant for her thorough studies and thank her for the privilege of discussing her paper.

DR. MARGUERITE A. CONSTANT (closing): I would like to thank Dr. Wachtl for his excellent discussion.

I regret I have no theory concerning the effect of ascorbic acid in the lens culture studies. As more criteria are brought into these studies perhaps some of these questions may become answered.

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# STUDIES ON THE CRYSTALLINE LENS*

## VII. UTILIZATION OF SEVERAL CARBOHYDRATES AND THEIR EFFECTS ON MITOSIS OF LENS EPITHELIUM

CARL WACHTL, PH.D., AND V. EVERETT KINSEY, PH.D.
*Detroit, Michigan*

Cataracts are known to develop in animals fed rations rich in galactose or xylose.[1-4] However, the mechanism of cataract formation associated with high levels of sugars is obscure.†

To gain further insight into the relation between carbohydrates and cataract formation, two investigations were undertaken. The first study was concerned with the relative rate of utilization of glucose, galactose, xylose, and fructose and the proportion of these sugars converted to lactate; the second dealt with the effect of the foreign sugars, alone or in combination with glucose, on the mitotic activity of the epithelial cells of the lens. Mitosis is a vital physiologic function, hence, measurement of the number of cells undergoing division may be presumed to be an index of the physiological state of the lens. This index was affected by apparently slight alterations in the composition of the culture media.[5]

### METHODS

#### CULTURE

Lenses were cultured under conditions and in media which, except for the presence of the foreign sugars, were believed to be physiologic. Lenses from albino rabbits weighing from 1.5 to 3.0 kg. were cultured at 37°C., pH 7.55, in an atmosphere of 88-percent nitrogen, seven-percent oxygen, and five-percent carbon dioxide. The technique and basic medium have been described elsewhere.[6]

For the present studies, the medium previously described was modified in two ways. In medium No. 1, 0.8-percent rabbit plasma was added. Medium No. 2 differed from medium No. 1 in the following respects: Alpha-ketoglutaric acid was replaced with

* From the Kresge Eye Institute. This work was supported in part by a grant from the United States Atomic Energy Commission.

† In the instance of diabetic cataract, it has been suggested[7] that the lack of insulin rather than the presence of excessive glucose in the blood is the causative factor.

glutamine, it contained TPN, and did not contain plasma or the six nucleosides present in the orginal medium. This medium is entirely synthetic and of known composition.

Medium No. 2, which supported a normal level of epithelial cells in mitosis in cultured lenses for nine hours, was used both for the studies of carbohydrate utilization (24-hour periods) and mitosis (nine-hour periods), while medium No. 1 was employed only for the former. Using a sterile technique, the following antibiotics were added per 5.0 ml. of media when lenses were cultured for 24 hours: potassium penicillin (200 units); dihydrostreptomycinsulfate (0.5 mg.); and n-butyl-paraphydroxybenzoate (1 gamma). All of the sugars were of the D-configuration.

### Analytic

| | |
|---|---|
| Glucose and galactose: | Anthrone procedure.[8] |
| Fructose: | Colorimetric method using one-percent resorcinol in concentrated hydrochloric acid.[9] |
| Xylose: | Determination of the color formed in sulfuric acid in the presence of cysteine.[10] |
| Lactic Acid: | p-hydroxydiphenyl method.[11] |

*Galactose in the presence of glucose.* The color produced by both galactose and glucose was first determined employing the anthrone procedure, and that produced by galactose alone was then measured after removal of the glucose by yeast fermentation. From a knowledge of the ratio of the extinction coefficients for galactose and glucose (0.52), the quantity of each of these sugars was then calculated.

Since small amounts of yeast still present in the solution following fermentation were found to interfere seriously with the anthrone method, the following procedure was employed to eliminate all traces of yeast: one ml. of the solution to be tested is transferred to a small flat-bottom test tube and two ml. of yeast suspension[12] are added. The tube is shaken laterally and incubated for 30 minutes at 37°C. The shaking is repeated every seven minutes. The suspension is then centrifuged for 20 minutes at 2,500 rpm, 0.5 ml. of the supernatant fluid is pipetted into another centrifuge tube and two ml. of five-percent trichloroacetic acid are added. The tube is stoppered, shaken, and centrifuged. One ml. of the above supernatant is used for determination of galactose concentration by the anthrone method.

*Xylose in the presence of glucose.* The xylose concentration was measured using Dische's method.[10] From a knowledge of the total color produced with anthrone, the concentration of glucose was determined.

The carbohydrate and lactate content of media were ascertained before and after culture, and from the differences in concentration, the amounts of carbohydrate consumed and lactate produced were computed.

*Mitotic counts.* Flat mounts of Feulgen-stained lens epithelia were prepared using the method of Howard[13] as modified by von Sallmann et al.[14] The total number of epithelial cells in all phases of division was determined in the epithelium of the cultured lens and compared with that in the uncultured contralateral lens. The results are expressed on a percentage basis in vitro with respect to in vivo.

## Results

The amount of glucose consumed and lactate produced in various culture media and for different concentrations of glucose are shown in Table 1* and Figure 1.† The glucose consumption of the lens is approximately the same in medium No. 1 and slightly higher in medium No. 2 than in aqueous humor. Increasing the glucose in the media from 125 mg. percent to 250 mg. percent has little effect upon the amount utilized. In all three media, about seven-tenths of the glucose consumed is converted to lactate.

Table 2 and Figures 2 and 4 show the

* Tables indicate individual data.
† The bar diagrams of figures signify averages.

TABLE 1

GLUCOSE CONSUMPTION AND LACTATE PRODUCTION PER RABBIT LENS FOR 24-HOUR PERIODS OF CULTURE

| Medium | Glucose Concentration | Glucose Consumed Per Lens | Lactate Produced | Lactate / Glucose |
|---|---|---|---|---|
| | | Mg. | Mg. | |
| Aqueous humor (rabbit) | Mg. % Approx. 125 | 4.2 | 1.9 | 0.45 |
| | | 4.2 | 2.6 | 0.63 |
| | | 3.8 | 3.1 | 0.82 |
| | | 3.9 | 3.4 | 0.87 |
| No. 1 | 125 | 4.4 | 3.0 | 0.68 |
| | | 5.5 | 3.3 | 0.60 |
| | | 4.4 | 2.2 | 0.50 |
| | | 4.3 | 3.4 | 0.79 |
| | | 4.5 | 1.8 | 0.40 |
| | | 4.4 | 2.7 | 0.62 |
| | | 4.8 | 2.6 | 0.54 |
| | | 4.6 | 4.2 | 0.91 |
| | | 4.5 | 3.9 | 0.87 |
| | | 3.9 | 2.7 | 0.69 |
| | | 4.7 | 3.2 | 0.68 |
| | | 3.5 | 2.5 | 0.71 |
| | | 3.5 | 3.1 | 0.89 |
| | 250 | 3.6 | 2.3 | 0.64 |
| | | 3.6 | 2.1 | 0.58 |
| No. 2 | 125 | 6.4 | 5.7 | 0.89 |
| | | 6.5 | 3.0 | 0.46 |
| | | 6.2 | 4.7 | 0.76 |
| | | 4.5 | 3.8 | 0.84 |
| | 180 | 4.6 | 3.4 | 0.74 |
| | | 4.8 | 3.6 | 0.75 |
| | | 4.0 | 3.6 | 0.90 |
| | | 6.7 | 3.8 | 0.57 |
| | | 6.3 | 2.9 | 0.46 |
| | | 5.3 | 2.2 | 0.42 |

amounts of galactose metabolized and the amounts of lactate produced in the presence and absence of glucose. The observations indicate that galactose is utilized less well than glucose, and unlike glucose (see table 1), the consumption increases with the concentration. The proportion of galactose converted to lactate was less than that for glucose. Like glucose, the consumption of galactose was slightly greater in medium No. 2.

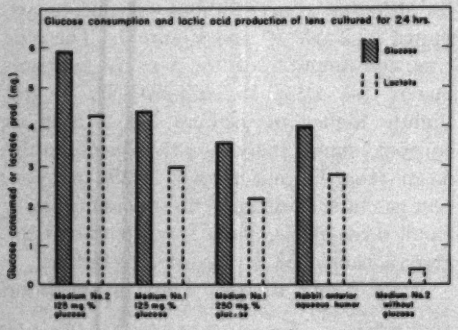


Fig. 1 (Wachtl and Kinsey). Glucose consumed and lactate produced in various culture media.

Table 3 and Figure 3 show that approximately the same amount of xylose was utilized as galactose. The amount of lactate recovered from lenses cultured in the presence of xylose did not exceed that found when lenses were cultured in the absence of any carbohydrate (fig. 1).

Table 4 and Figure 3 show that fructose was not well utilized when present at a concentration similar to that of glucose in aqueous humor, but the amount consumed increased considerably with concentration.

TABLE 2

GALACTOSE CONSUMPTION AND LACTATE PRODUCTION PER RABBIT LENS IN THE PRESENCE OR ABSENCE OF GLUCOSE FOR 24-HOUR PERIODS OF CULTURE

| Medium | Galactose Concentration | Glucose Concentration | Galactose Consumption | Glucose Consumption | Lactate Produced | Lactate / Carbohydrate |
|---|---|---|---|---|---|---|
| | Mg. % | Mg. % | Mg. | Mg. | Mg. | |
| No. 2 | 125 | 0 | 2.1 | — | 1.3 | 0.62 |
| | | | 1.8 | — | 1.1 | 0.61 |
| | 250 | 0 | 3.4 | — | 1.7 | 0.50 |
| | | | 3.3 | — | 1.5 | 0.45 |
| | | | 2.9 | — | 0.8 | 0.28 |
| No. 1 | 250 | 0 | 2.5 | — | 1.1 | 0.44 |
| | | | 2.5 | — | 1.1 | 0.44 |
| | 62½ | 62½ | 1.5 | 2.9 | 3.5 | 0.80 |
| | | | 1.2 | 2.4 | 3.3 | 0.92 |
| | | | 1.2 | 3.2 | 3.8 | 0.86 |
| | | | 1.1 | 2.5 | 2.6 | 0.72 |
| | 125 | 125 | 2.1 | 2.8 | 4.5 | 0.92 |
| | | | 1.5 | 2.3 | 3.5 | 0.92 |
| | | | 2.2 | 3.1 | 4.9 | 0.92 |

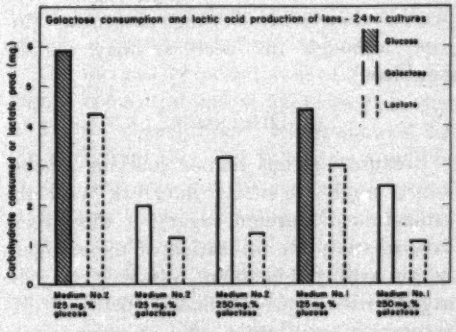
Galactose consumption and lactic acid production of lens - 24 hr. cultures
Glucose
Galactose
Lactate
Carbohydrate consumed or lactate prod. (mg.)
Medium No.2 125 mg.% glucose
Medium No.2 125 mg.% galactose
Medium No.2 250 mg.% galactose
Medium No.1 125 mg.% glucose
Medium No.1 250 mg.% galactose

Fig. 2 (Wachtl and Kinsey). Galactose metabolism.

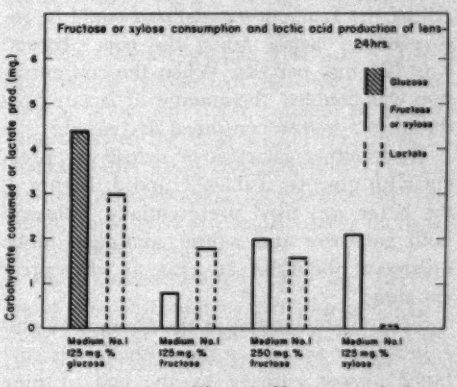
Fructose or xylose consumption and lactic acid production of lens - 24hrs.
Glucose
Fructose or xylose
Lactate
Carbohydrate consumed or lactate prod. (mg.)
Medium No.1 125 mg.% glucose
Medium No.1 125 mg.% fructose
Medium No.1 250 mg.% fructose
Medium No.1 125 mg.% xylose

Fig. 3 (Wachtl and Kinsey). Xylose metabolism.

TABLE 3

XYLOSE CONSUMPTION AND LACTATE PRODUCTION PER RABBIT LENS IN THE PRESENCE OR ABSENCE OF GLUCOSE FOR 24-HOUR PERIODS OF CULTURE

| Medium | Xylose Concentration | Glucose Concentration | Xylose Consumption | Glucose Consumption | Lactate Produced | Lactate / Carbohydrate |
|---|---|---|---|---|---|---|
| | Mg. % | Mg. % | Mg. | Mg. | Mg. | |
| No. 2 | 125 | 0 | 2.4 | — | 0.2 | 0.1 |
| | | | 1.7 | — | 0.0 | 0 |
| | 62½ | 62½ | 1.7 | 2.2 | 1.2 | 0.31 |
| | | | 1.3 | 2.6 | 2.5 | 0.64 |
| | | | 0.9 | 3.1 | 2.2 | 0.55 |

TABLE 4

FRUCTOSE CONSUMPTION AND LACTATE PRODUCTION PER RABBIT LENS IN THE ABSENCE OF GLUCOSE FOR 24-HOUR PERIODS OF CULTURE

| Medium | Fructose Concentration | Fructose Consumed | Lactate Produced | Lactate Fructose |
|---|---|---|---|---|
| | Mg. % | Mg. | Mg. | |
| No. 1 | 125 | 0.7 | 1.6 | 2.3 |
| | | 0.7 | 2.0 | 2.9 |
| | | 1.1 | 1.7 | 1.5 |
| | 250 | 2.1 | 1.9 | 0.90 |
| | | 2.4 | 1.6 | 0.67 |
| | | 1.5 | 1.4 | 0.93 |
| No. 2 | 125 | 0.6 | 1.8 | 3.0 |
| | | 0.7 | 0.7 | 1.0 |
| | | 0.7 | 1.7 | 2.4 |
| | | 0.7 | 0.8 | 1.1 |
| | 250 | 1.7 | 1.9 | 1.1 |
| | | 2.1 | 1.9 | 0.90 |
| | | 1.7 | 1.6 | 0.94 |

All of the fructose metabolized could be recovered as lactate when the concentration was 125 mg. percent. When the concentration was doubled, the amount of lactate produced to fructose consumed decreased.

When either galactose or xylose was present with glucose, Tables 2 and 3, Figure 4, the latter was used preferentially, although both galactose and xylose continued to be utilized at about the same rate as when present alone.

Table 5 shows that in the absence of glucose mitotic activity in the epithelium is curtailed almost entirely by the end of nine

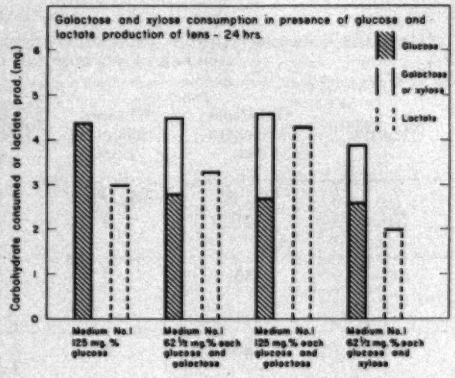
Galactose and xylose consumption in presence of glucose and lactate production of lens - 24 hrs.
Glucose
Galactose or xylose
Lactate
Carbohydrate consumed or lactate prod. (mg.)
0
1
2
3
4
5
6
Medium No.1 125 mg. % glucose
Medium No.1 62½ mg.% each glucose and galactose
Medium No.1 125 mg.% each glucose and galactose
Medium No.1 62½ mg.% each glucose and xylose

Fig. 4 (Wachtl and Kinsey). Galactose and xylose metabolism.

hours of culture. Table 5 also shows that the other sugars tested, namely, galactose, fructose, or xylose can only support mitosis at a small fraction of the normal rate. The mitotic activity appeared to be reduced slightly when any of the three foreign sugars were present along with 62.5 mg. percent of glucose, although the decrease may not be significant.

## DISCUSSION

Because aqueous humor contains glucose which might in itself interfere with the utilization of foreign sugars, it was necessary to study the utilization of these sugars in an artificial medium. While it may be argued with some justification that any difference in utilization of a foreign sugar

TABLE 5

MITOTIC COUNTS IN LENS EPITHELIA AFTER NINE-HOUR CULTURE IN MEDIA CONTAINING VARIOUS CARBOHYDRATES EXPRESSED AS PERCENTAGE OF THE CONTROL EYES

| Carbohydrate Present | Concentration Mg. % | Number Lenses | In Vitro/In Vivo | |
|---|---|---|---|---|
| | | | % | Range |
| *None | — | 4 | 4 | (1–7) |
| Glucose | 125 | 9 | 84 | (46–110) |
| Galactose | 125 | 4 | 2 | (0–4) |
| Fructose | 125 | 4 | 4 | (0–9) |
| Xylose | 125 | 4 | 10 | (1–32) |
| Galactose and Glucose | 62½ each | 6 | 37 | (4–85) |
| Fructose and Glucose | 62½ each | 4 | 52 | (9–77) |
| Xylose and Glucose | 62½ each | 3 | 52 | (30–64) |

* K.E.I. Medium No. 2 without glucose was used in this experiment.

compared with glucose might be due to the inadequacy of the media, rather than to inability of the lens to metabolize the sugar, the observation that similar amounts of glucose were consumed in aqueous humor and in the two media employed in the present study suggests that the media used are adequate to determine relative rates of utilization. However, it has been shown previously[5] that of the two artificial media, only medium No. 2 supported a normal number of epithelial cells in mitosis; hence, only this medium was employed for testing the effects of the sugars on mitotic activity.

When fructose was present in the media at a concentration of 125 mg. percent, the lactate-fructose ratio was about two, thus, other constituents of the lens or the media must have been converted to lactate. This observation suggests that fructose, when present in concentrations analogous to those of glucose in aqueous humor, does not in itself provide sufficient energy to meet the metabolic requirements of the lens.

The increased consumption of fructose at higher concentrations is interesting in view of Harris' observations[15] which showed that the potassium recovery following a chilling of the lens was but 30 percent of normal at fructose levels of 200 mg. percent, but increased to 84 percent at 500 mg. percent. The dependence of fructose utilization on concentration can no doubt account for the observations of Suellmann,[16] who found that lenses provided with fructose maintained the same concentration of phosphoric acid esters as those supplied with glucose. However, Suellmann employed extremely high concentrations of fructose, 2,500 and 5,000 mg. percent, which may have enabled the lens to provide the necessary energy for normal levels of phosphoric esters.

Failure of xylose to be converted to lactate in appreciable amounts confirms unpublished results of von Heyningen who studied xylose metabolism in calf lenses.[17]

Although the lens utilizes all of the foreign sugars to some extent, it seems doubtful, on the basis of studies of mitosis, that sufficient energy resulting from their breakdown is made available for cell function.

## Summary

The amount of glucose, galactose, xylose and fructose utilized, the amount of lactate formed, and the ability of these sugars to maintain mitosis in the epithelium of the lens of the rabbit have been investigated following culture in a medium and under conditions which are thought to maintain the lenses in a normal physiologic state.

Lenses cultured in the artificial media employed metabolize from three to five mg. of glucose per day, or slightly more than they do when cultured in rabbit aqueous humor. Of this amount, approximately two thirds is converted into lactate. The quantity of glucose consumed is not altered when the concentration in the media is raised from 125 mg. to 250 mg. percent.

Lenses metabolize galactose and xylose at approximately one half the rate, and fructose at about one fifth the rate of that of glucose when these sugars are present in the media at the same concentration (125 mg. percent). In contrast to glucose, the consumption of galactose and fructose increases with the concentration.

At a concentration of 125 mg. percent, about one half of the galactose and essentially all of the fructose are converted to lactate, whereas little, if any, lactate is produced from xylose.

The utilization of galactose, xylose, and fructose is unaffected by the presence of glucose in the medium and the glucose metabolism continues unaffected by the foreign sugars.

None of the foreign sugars is capable of supporting appreciable mitotic activity of the lens epithelium for periods of nine hours. Their presence along with the glucose appears to depress somewhat the proportion of cells undergoing mitosis.

*690 Mullett Street (26).*

Technical assistance was given by Miss Jean Gorman.

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## Discussion

Dr. Bernard Schwartz (Iowa City, Iowa): In considering the discussion of this paper I thought it would be worthwhile at this time to consider briefly some of the factors that must be taken into account when any tissue is cultured in vitro. One of the first things to be done is to mimic physiologic conditions as closely as possible. The authors have succeeded in accomplishing this in their persistent efforts to obtain a synthetic medium akin to the aqueous humor. They have also maintained the oxygen and carbon dioxide tensions as well as the pH at physiologic levels.

However, there is one aspect of their culture technique which, to my mind, is quite "unphysiologic" and that is the use of the batch method. The batch method involves the complete change of the culture fluid at various intervals. This method was originally chosen by Dr. Kinsey in preference to a perfusion technique in order to measure easily changes in the metabolic constituents of the culture fluid. The disadvantages of the batch method as Dr. Kinsey pointed out in his first paper are the accumulation of metabolites and the exhaustion of substrate with the possibility of pH shifts of the culture fluid. Thus there is a continually changing level of metabolites and substrate throughout the culture period in the batch method. This is in contrast to the steady-state conditions that exist in the eye with the continuous flow of aqueous humor past the lens. One wonders whether, in the batch system, the metabolism of the lens is continuously changing and is not at any steady-state level. An analogy in the field of microbiology may make this point slightly clearer.

We are all familiar with the batch method of growing bacteria in large flasks. The growth curve of these organisms usually consists of three phases —a lag phase, a "log" phase, and a steady period followed by a decline. It is well known to microbiologists that the metabolism and morphology of the organisms varies with the phase of their growth. Similar observations have recently been reported in tissue culture in the study of cell suspensions.

In order to avoid these continually changing conditions of the medium due to changes in concentration of substrates and metabolic end-products, some microbiologists have turned to the use of continuous culture techniques whereby both steady state levels of populations and metabolism are obtained. It is my belief that in lens culture a more physiologic technique would be obtained by using a perfusion system and our efforts are being directed

at the moment toward this goal.

The second problem to be considered is that of selecting criteria of physiologic normalcy for the cultured tissue. The authors have shown in previous studies that the mitotic activity of the lens epithelium is a sensitive index. Changes in the mitotic count occur before disturbances of transparency, the level of ascorbic acid, or the sodium turnover in a damaged lens. On the basis of the mitotic count as the physiologic normal for the whole lens the authors have successfully developed a synthetic medium. Now the question arises in my mind as to whether the mitotic count does actually reflect normalcy for the whole lens or only for the epithelium. alone. It is conceivable that other metabolic functions in the lens fibers, such as protein metabolism, could be diverted to supplying energy to maintain a normal rate of mitosis in the epithelium. In this paper the authors have partially guarded against this possibility by measuring glucose consumption and lactic acid production as well as the mitotic count.

The third problem to be considered is especially concerned with the role of permeability in the metabolism of a substrate by a tissue in culture. It is interesting to speculate if the decreased utilization of galactose, xylose, and fructose as compared with glucose is due to a permeability factor. It is known that the intestinal absorption coefficients (mg. of sugar absorbed/100 gm. of rat liver/hour) are: galactose greater than glucose greater than fructose greater than xylose. Also Van Heyningen has shown with the intact calf lens that galactose and xylose compared to glucose penetrate poorly. The permeability factor thus could explain the differential and decreased utilization of these monosaccharides. The differences in lactate production indicate differences in metabolic pathways or rates of breakdown. The fact that none of the monosaccharides supported mitotic activity may be due to a combination of the permeability factor and differences in metabolic pathways and rates, so that insufficient energy is produced to sustain normal mitotic rate.

The authors are to be complimented on their continuing efforts to study lens metabolism in vitro.

DR. ZACHARIAS DISCHE (New York): I think there is one interesting aspect in the data presented by Dr. Wachtl, and that is that apparently the decrease in mitosis has nothing to do with the utilization of carbohydrates like galactose and fructose exciters which depress the mitotic rate.

I wonder if this is due to the fact that we must discern between the utilization of these sugars by the lens as a whole, and the utilization by the epithelium itself, and whether there is not a great difference between these two processes.

The problem appears to have some interest because, as was shown quite recently by Schwartz and Goldman in England, in rats kept on a high galactose diet there is a very high accumulation of galactose-1 phosphate in the capsule and in the epithelium of the lens, but not in the lens fibers themselves.

These authors have shown in studies on red cells of infants with congenital galactosemia that the accumulation of the galactose-1 phosphate interferes with the utilization of glucose.

DR. CARL WACHTL (closing): I would like to emphasize that the analyses we have made showed that the levels of a number of physiologically important compounds were unchanged after 24 hours of culture at which time the medium is replaced by fresh medium. Moreover, because of the buffering capacity of the medium, the pH does not change appreciably during culture. These facts seem to indicate that alterations which occur in the content of metabolites do not disturb the steady state of the lens.

As far as I know, most of our knowledge of bacterial growth was obtained from culture studies without the application of the perfusion technique. I am certain that by the use of this method, additional information can be gained. The perfusion technique has often been applied in the past when others failed. For instance, a reason for its application was to provide cultured organs with a continuous supply of blood.

The suggestion of Dr. Schwartz to use other methods which would give information concerning the lens fibers is very sound. In fact, we have been trying to make tissue sections of the lens for that purpose. The mitotic count does not by itself supply the only important information. It is one of the tests, and it seems to be quite a sensitive one, by which the adequacy of a medium for the culture of the lens can be ascertained.

I firmly agree with the tremendous importance of permeability. Dr. Davis of New York University called attention to this fact in his talk on permeability and enzyme activity in bacteria. When we add a substance to the medium, we do not know whether it enters the lens unless we test for it.

Concerning the remarks of Dr. Dische, it is true that the factors which control mitosis and glucose consumption are in general not identical, though mitosis stops in the absence of glucose. In 1940, Dr. Willmer working with chick fibroblasts could not find any correlation between mitotic activity on the one hand and glucose consumption or lactate production on the other. Dr. Dische pointed out quite correctly that one should perhaps distinguish between utilization by the epithelium and by the lens. It is quite probable that galactose-1-phosphate does interfere with the metabolism of glucose.

I would like to thank Dr. Schwartz for his discussion of our paper and Dr. Dische for his remarks.

# THE REACTIVITY OF THE SULFHYDRYL GROUPS IN NORMAL BOVINE LENS*

LORENZO O. MEROLA, B.S., AND JIN H. KINOSHITA, PH.D.
*Boston, Massachusetts*

## INTRODUCTION

One of the most widely studied chemical aspects of the cataractous process is the change in the sulfhydryl groups (-SH).[1-6] There seems to be general agreement that in experimental cataract formation, the amount of lens glutathione drops markedly before any sign of opacification. On the other hand, as evidenced by irradiation cataract studies of Pirie and Van Heyningen,[3] the loss of protein -SH (P-SH) is observed at a much later stage in the formation of a cataract. These results suggest that the reactivity of the -SH of glutathione (GSH) is different from that of lens protein.

The disappearance of the -SH groups seems to involve an oxidation resulting in the formation of disulfide linkages as demonstrated by Dische and Zil.[4] These investigators also suggest that the oxidation of -SH occurs not only in the cataract process but also in the transformation of soluble to the insoluble protein.

In view of the many studies on the change of -SH levels in the cataractous lens, it was felt that an investigation on the factors which influence the reactivity of -SH groups of normal lens would be worth while. In this study, the ability of the -SH groups to undergo oxidation was used as the measure of their reactivity. This index of reactivity seems particularly appropriate since the oxidation of -SH groups appears to play an important role in establishing the state of lens protein.

## METHODS

### PREPARATION OF LENS HOMOGENATES

Lenses were removed from the eyes of freshly slaughtered cattle and were washed with glass redistilled water. After removal of excess water by blotting with filter paper, the lenses were weighed and then homogenized in a Waring blender at 5°C. under nitrogen in isotonic Tris† buffer at pH 7.4. The amount of buffer used for homogenization was to give a final dilution of one lens in 20 cc. of buffer. The insoluble material of the lens was removed by centrifugation at 10,000 × g for 15 minutes at 5°C.

### PROCEDURE FOR LENS -SH OXIDATION STUDIES

In an Erlenmeyer flask, which was shaken in a Warburg bath, 20 cc. of the homogenate containing the soluble lens protein was incubated at 25°C. or 37°C. in the presence of cupric ion ($2 \times 10^{-5}$M). The flask was flushed for 10 minutes with 100-percent oxygen and then clamped off. An aliquot of the homogenate was removed every hour and the total -SH was determined by amperometric titration (described below). The GSH was measured on a protein free filtrate by the nitroprusside method.[7] In some cases, the amperometric titration method was used. In either case both methods gave the same values. The oxidized glutathione was determined by the electrolytic reduction method previously described.[7] The method of Roe[8] was employed for the determination of ascorbic acid.

### AMPEROMETRIC TITRATION

The lens P-SH groups were determined by the difference between the amount of total -SH obtained by amperometric titration and the GSH present. The amperometric titration employed was the method of Benesch

* From the Howe Laboratory of Ophthalmology, Harvard Medical School. This work was supported in part by the United States Atomic Energy Commission, and by a Graduate Training Grant P.H.S. No. 2-B-5096 from the National Institute of Neurological Diseases and Blindness, Public Health Service.

† Tris (hydroxymethyl) aminomethane.

et al.[9] with slight modifications. An external potential was applied (−0.10v.) and the reference electrode was a mercury pool. A more detailed description of this method with the modifications will be presented in a future publication. The titration was carried out in 100 cc. electrolytic beaker containing 30 cc. of a supporting electrolyte made up by 4.0 cc. of 1.0M Tris, 3.4 cc. of 1.0M $HNO_3$, and 0.3 cc. of 1.0M $KNO_3$. To this media a solution containing approximately 1 μM of -SH was added and titrated with $1 \times 10^{-3}$M $AgNO_3$ solution. To prevent possible oxidation during the titration nitrogen was bubbled into the titrating mixture. However, when the galvanometer readings were made, the bubbling was stopped to prevent disturbances of the diffusion current. During this short interim nitrogen was passed over the titrating mixture. The apparatus and procedure were checked every day by titrating standard amounts of GSH. To demonstrate that certain substances of the lens homogenate did not interfere with this procedure, known quantities of GSH were added to the homogenate and titrated. A 99 to 100-percent recovery was obtained in all cases.

## Results and Discussion

As can be seen in Table 1, considerable quantities of -SH groups are found in bovine lens. The total -SH content is 54.1 μM per gm. wet weight of lens. Of this amount 9.4 μM is GSH and 44.7 μM is P-SH.

As mild as possible oxidizing conditions were employed to study the factors which influence the oxidation of -SH groups of lens. If strong oxidizing agents such as hydrogen peroxide are used, the oxidation is so rapid that it becomes difficult to demonstrate the differences in the reactivity of the various -SH groups. On the other hand, if simply an atmosphere of oxygen is used then the -SH oxidation is so slow that it becomes impractical to study. In the experiments to be reported here, the conditions employed were to catalyze the oxidation with $2 \times 10^{-5}$M cupric ion and to use 100 percent $O_2$ as the gas phase.

TABLE 1

Sulfhydryl content of bovine lens

| No. of Observations | Type of -SH groups | μM-SH/gm. lens (wet) |
|---|---|---|
| 15 | Total -SH | 54.1 ± 3.6 S.D. |
| 15 | Protein -SH | 44.7 ± 3.35 S.D. |
| 15 | Glutathione (Reduced) | 9.4 ± 0.56 S.D. |

From the preliminary experiments it was apparent that in the intact lens the level of -SH groups of protein and GSH did not change significantly when lens was incubated at neutral reaction under the oxidizing conditions described above. Therefore, it was necessary to use homogenized lens to study the factors which influence the oxidation of -SH groups in lens. In Figure 1, the extent of the disappearance of the -SH groups of lens homogenate is shown. In contrast to the findings with the intact lens, homogenization of the lens and subsequent incubations under conditions favorable for oxidation, resulted in a rapid disappearance of GSH. The effect of the presence of catalytic amounts of cupric ion is demonstrated in the oxidation of lens GSH. In the presence of cupric ion the disappearance of GSH is complete within one hour, but without added cupric ion five hours are required for complete oxidation. In both cases the disappearance of GSH seems to be due to its oxidation to the disulfide form since most of it is recovered when the protein-free filtrate is subjected to electrolytic reduction.

In a previous paper[7] from this laboratory, evidence was presented suggesting the possibility that GSH in the intact lens appears to be bound in some manner. From the studies on the extractability of GSH it appeared that this peptide was not freely diffusable, and from the effect of concentrated urea and guanidine solutions it was suspected that binding of GSH involves hydrogen bonds. The fact that in the intact lens

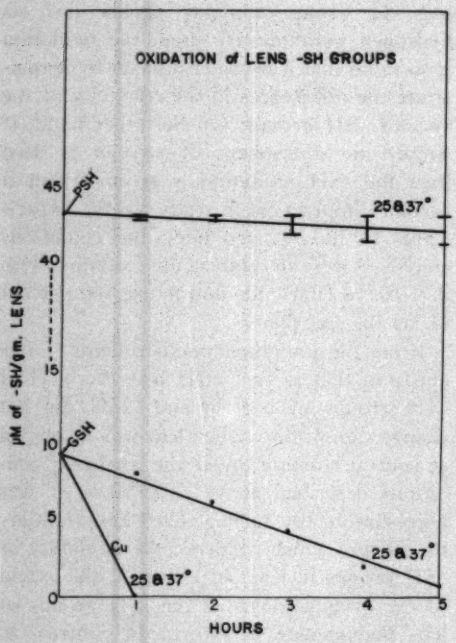


Fig. 1 (Merola and Kinoshita). Oxidation of lens sulfhydryl groups. Lens homogenates were incubated with and without $2 \times 10^{-5}$M cupric ion in the presence of 100-percent oxygen.

only a slight amount of GSH is capable of being oxidized is consistent with the hypothesis that GSH is bound in lens. For if it were freely diffusible, oxidation, which requires the collision and interactions of two molecules of GSH, would take place. In the intact lens this does not occur to any significant extent. When the lens is homogenized, however, the GSH being a tripeptide not having many sites to establish strong hydrogen bonding is released from these weak binding sites and is able to undergo oxidation.

In contrast to the ease with which GSH is oxidized, the P-SH groups, even in lens homogenates, are particularly resistant to the oxidizing conditions employed. As is shown in Figure 1, the level of P-SH in the presence of cupric ion does not change significantly. In five hours at 25°C. or 37°C. no more than seven percent of the P-SH groups disappears. It, therefore, appears that the lens P-SH groups are not easily susceptible to oxidation.

A noteworthy fact emerges from this experiment. GSH and ascorbic acid have been considered by many as serving some type of protective action on the P-SH groups.[2] It is believed that these reducing substances, found in such high concentrations in lens, help maintain the -SH groups of lens protein in the reduced state. The evidence from our experiments does not seem to support this idea. From Figure 1, it can be seen that in the copper catalyzed homogenates, the level of P-SH is still maintained even after all the GSH is oxidized. The experiments with resin-treated homogenates also indicate that GSH does not participate in maintaining the P-SH level. For this study, lens homogenates were passed through a column of Dowex 1 on a nitrate cycle. The GSH was removed completely by this treatment but no loss in the P-SH was observed. Incubation of the resin-treated homogenates under the oxidizing conditions for five hours resulted only in a slight decrease in the amount of P-SH content (five percent). These studies are further supported by the experiments on dialyzed lens homogenates. In these experiments, lens homogenates were dialyzed against 80 volumes of isotonic potassium nitrate for 24 hours at 5°C. The dialysis was effective in removing all the GSH and ascorbic acid as checked by colorimetric tests. Dialysis under these conditions did not change the amount of P-SH which is further indication of the stability of this group. The dialyzed homogenates when incubated at 25°C. and 37°C. and subjected to the oxidizing conditions revealed a P-SH oxidation curve which was practically identical as the one observed in the experiment with undialyzed homogenates (fig. 1). From these results it would appear that the P-SH level of dialyzed lens homogenates does not change for five hours when exposed to the oxidizing conditions of these experiments. Therefore it seems that the absence of GSH or ascorbic acid does in no way influence the level of lens P-SH.

It would appear that the reason why the lens P-SH are so unreactive is that they are "masked." The "masking" is thought to be due to hydrogen bonding between the sulfhydryl groups and neighboring atoms. These bonds have been demonstrated to be broken by high concentrations of compounds which also have a strong tendency to form hydrogen bonds. The most effective of these reagents are urea and guanidine solutions.[10,11] To demonstrate the "masked" state of the lens P-SH, lens homogenate was treated with 8.0M urea solutions and subjected to the oxidizing conditions. The results of incubation studies at 25°C. and 37°C. are shown in Figure 2. It appears that the presence of 8.0M urea has a marked effect on the reactivity of the P-SH. At both temperatures there is an increase disappearance of P-SH. As might be expected, the rate of disappearance at 37°C. is much faster than 25°C. We believe that the disappearance of the -SH groups is due to an oxidative process probably involving the formation of disulfide bonds. The evidence for this is that the disappearance of the P-SH and GSH is prevented when $O_2$ is replaced by $N_2$. The determination of $O_2$ uptake by dialyzed lens homogenate is now being undertaken. A few preliminary studies have revealed that when 8.0M urea is added to the dialyzed homogenate, there is an increase in $O_2$ consumption concomitant with the disappearance of P-SH.

The increase in reactivity of P-SH by urea treatment indicates that the disruption by hydrogen bonds has brought about the "unmasking" of the -SH groups. Other investigations appear in the literature which support the findings that lens P-SH are "masked." Nordmann et al.[12] have shown that the $O_2$ consumption of boiled lens homogenate is much higher than unboiled homogenate. The $O_2$ consumption is inhibited by lead acetate presumably by tying up the "unmasked" -SH. Heat denaturation, although not as effective as urea treatment, is another means by which lens P-SH may be "unmasked." Another investigation which

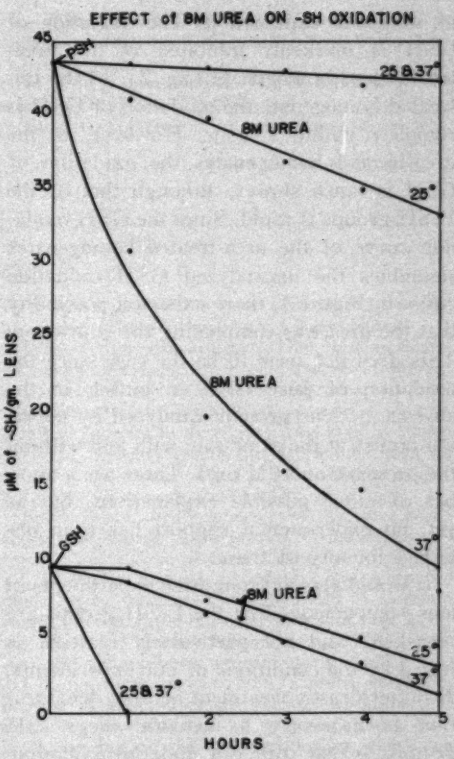


Fig. 2 (Merola and Kinoshita). Effect of 8.0 M urea on sulfydryl oxidation. Lens homogenates were incubated with $2 \times 10^{-5}$M cupric ion in the presence of 100-percent oxygen.

may be cited is that by Gloster[13] who studied the reduction of dehydroascorbic acid in lens extract. In attempting to show that the reduction of dehydroascorbic acid was not due to an enzymatic process, he presented evidence that boiled lens extract reduced dehydroascorbic acid more effectively than did the untreated lens extract. His observation may be explained by the fact that heat denaturation has served to increase the reactivity of the lens P-SH to reduce dehydroascorbic acid. In our experiments, we were also able to demonstrate the reduction of dehydroascorbic acid by urea-treated lens homogenates.

In the incubation studies on the urea-treated homogenates, an observation on the GSH oxidation was made for which we have

no adequate explanation. The oxidation of GSH is markedly inhibited in the urea-treated lens homogenate (fig. 2). In the untreated homogenate the oxidation of GSH is complete within 1 hour. However, in the urea-treated homogenates the oxidation of GSH is much slower, although that of the P-SH groups is rapid. Since the GSH oxidation curve of the urea-treated homogenates resembles the uncatalyzed GSH oxidation curve in Figure 1, there existed a possibility that the urea was complexing the cupric ion. This does not seem to be the case since the oxidation of pure GSH in buffer, in the absence of lens protein, catalyzed by cupric ion occurs at the same rate with and without the presence of 8.0M urea. There are a number of other possible explanations, but as yet, no experimental support has been obtained for any of these.

It would appear from these experiments in lens homogenates that the P-SH groups are "masked" and not particularly reactive as tested by the conditions of our experiments. A rather drastic treatment of urea denaturation is necessary to activate these -SH groups so that they can undergo oxidation. In contrast to these experimental observations, in cataract formation the conditions apparently become favorable so that the hydrogen bonds which "mask" the -SH groups are broken and the oxidation of these groups readily takes place. These facts suggest that in either cataracts or in lens of aged animals, where an oxidation of sulfhydryl groups occurs, there must first be a denaturation process which unfolds the peptide chains of lens protein so as to facilitate the oxidation.

## Summary

The level of P-SH groups in normal bovine lens as determined by amperometric titration was found to be 44.7μM -SH per gm. lens (wet weight). This concentration of P-SH is approximately four times that of GSH.

In the intact lens both the P-SH and GSH appear to be unreactive toward oxidation. The difference in reactivity of the -SH groups of protein and glutathione becomes apparent in the study of lens homogenate. The GSH is readily oxidized to the disulfide form. On the other hand the P-SH groups appear to be quite stable toward oxidation.

Compounds such as ascorbic acid and GSH which are dialyzable or removed by ion exchange seem to be unnecessary to maintain the level of P-SH groups.

The effect of concentrated urea solution on the reactivity of the -SH groups of protein seem to indicate that in the normal state these groups are marked.

*243 Charles Street (14).*

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## Acknowledgment

We wish to express our gratitude to Dr. W. Morton Grant and Dr. Charles Rife of the Howe Laboratory, for their advice and counsel on the problems of electrochemistry.

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## Discussion

Dr. Zacharias Dische (New York): I was very much interested in this impressive work which was carried out with such refined analytic procedures, and I was particularly interested because for the last few years our laboratory was carrying on investigations on the oxidation of lens proteins. We tried to obtain evidence that the change in certain physical or chemical factors in the lens might be responsible for an increase in the oxidation of the proteins.

We approached this problem in a somewhat different way than Dr. Merola and Dr. Kinoshita. They were apparently intent upon creating conditions which could be regarded as physiologic, that means to obtain oxidation of sulfhydryl groups by a catalyst.

Dr. Croisy, in our laboratory, tried something similar, although not on such a simplified system, by investigating the effect of phosphate on the oxidation of the sulfhydryl groups of rat lens proteins. Phosphate forms complexes with heavy metals, and phosphate solutions are a well-known oxidizing agent. Our results, which we presented about a year and a half ago at the eastern regional meeting of this association, agreed to a certain extent with the results of Dr. Merola and Dr. Kinoshita, in so far as we obtained 10 to 15 percent oxidation of the total soluble protein of rat lenses with phosphate. One possible objection that could be made against this type of experiments in which catalysts are used, is that the catalyst could be bound by proteins (as is very probable in the case of copper), and of course in this case, if the catalyst is bound by proteins, then we cannot be sure that the true availability of the sulfhydryl groups (if they are partly masked) can be determined, because what then determines the amount of oxidation is not only the availability of the sulfhydryl groups but also the availability of the catalyst for oxidation.

As we do not know the nature of the catalyst that is active in the lens, we may be completely misled.

For this reason, in collaboration with H. Zil, we were also trying to determine the availability of the sulfyhydryl groups against oxidizing agents which were present in excess, a type of experiments which Dr. Merola and Dr. Kinoshita also apparently tried, using hydrogen peroxide as the oxidizing agent in excess. In our experiments Potassium ferricyanide and $H_2O_2$ were used at pH 7.0 and above and Ferrichloride at pH 6.0 and below.

When experiments are carried out, with an excess of oxidizing agents, the results are very different as far as lens protein of cattle are concerned from those presented by the authors, because if we oxidize lens proteins by ferricyanide or even hydrogen peroxide, or heavy metals like ferric ions, then we obtain at physiological pH a much higher availability of SH groups, particularly at 37°C.

Furthermore, we have evidence that this availability can change as the process of oxidation proceeds. This was particularly clearly shown by Dr. Zil, in our laboratory, who found that when the oxidation by ferric ions has proceeded to a certain extent, then it stops, but after a certain time interval more SH groups become available to ferric ions and the oxidation process starts again.

I do not think we can reject, a priori, such phenomena as unphysiological, because we know that in the lens the oxidation of sulfhydryl group proceeds far beyond the few percentages which can be demonstrated by using catalysts. We must, therefore, rather assume that the availability of SH groups of lens proteins in vivo may be of the same order of magnitude as is observed against oxidizing agents present in excess of sulfhydryl groups. That means that secondary availability may be created by the oxidative process.

We tried also to increase the availability of proteins by the same procedures that the authors used, namely, by high concentration of urea and also some other agents, and we arrived at similar conclusions, although I must say that even at 25°C. we obtained a much higher increase in availability by urea than appeared in experiments of the authors. In my opinion this is probably due to very strong interactions between the catalyst and the lens protein on the one side, and the denaturing agent on the other side.

We must consider the possibility of the formation of mixed chelates between the copper, the protein and the urea, which may completely change the course of oxidation. Phenomena of this kind may be responsible for some of the very interesting observations reported in this paper.

I would like to express my great appreciation of the work done by the authors. It is very elegant, clear, and conclusive.

Dr. Lorenzo O. Merola (closing): May I thank

Dr. Dische for his very fine discussion.

In answer to Dr. Dische's first question. We believe that in our system free cupric ion was present in sufficient quantities to have a catalytic effect. For if this were not the case, then we should not have obtained 100-percent oxidation of glutathione within one hour where cupric ion was used, while, without cupric ion present, it took five hours to obtain approximately the same amount of oxidation.

Oxidation of protein sulfhydryl groups was used to determine whether they were free or masked by hydrogen bonding. When strong oxidizing agents, such as peroxide, were used the degree of protein-SH oxidation was dependent upon the concentration of the oxidizing agent. This suggested that this type of oxidizing agent appears capable of breaking hydrogen bonds, and, therefore, do not necessarily give a true index of the availability of the normal protein sulfhydryl groups. We felt that by using mild oxidizing conditions, the protein-SH groups would not be oxidized unless they were free or until they were made available by treatment with urea.

Again may we thank Dr. Dische for his fine discussion.

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# STUDIES ON PROTEIN AND PROTEIN SYNTHESIS*

## During the development of galactose cataract

Zacharias Dische, M.D., Ginevra Zelmenis, M.D., and Joshua Youlus, M.D.
*New York*

Previously,[1] it has been reported that when six-week-old rats are left for 21 days on a galactose diet inducing cataract, the total amount of soluble proteins as well as their concentration at the end of the feeding period is significantly lower than that of controls on normal diet, and about the same as it was at the beginning of the feeding period. This was interpreted as an inhibition of the synthesis of soluble proteins in rats on galactose diet.

It has been, furthermore, shown that feeding of galactose induces characteristic changes in the composition of the soluble proteins. These changes consist in an accumulation of a protein which, after oxidation with ferricyanide a pH 7.4 at 37°C., becomes insoluble, and precipitates. This protein which will be referred to as precipitable protein was also shown to be present in smaller quantities in lenses of young rats on normal diet. Its amount in these cases ran parallel to the rate of protein synthesis, so that it became insignificant at the end of the period of rapid growth of the lens. These observations suggested that this protein is an intermediate in the synthesis of lens proteins.

* From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University.

As there is evidence that the steady-state of soluble proteins in the lens is the result of the superimposition of a continuous breakdown of proteins on a process of synthesis, the inhibition of the net synthesis could be due to an increase in the rate of the first, as well as to a decrease in the rate of the second process.

In an attempt to reach an understanding of the mechanisms involved in the formation of galactose cataract, it seemed first of all necessary to determine whether the inhibition of the net protein synthesis and the accumulation of the abnormal protein appears immediately after the beginning of galactose feeding, and whether the degree of inhibition and accumulation changes significantly during the period of feeding. It seemed, furthermore, of particular interest to obtain some indications as to the nature of the abnormal protein which precipitates after oxidation, and whether such an abnormal protein can also be found in lenses of other young animals.

We determined, therefore, the net synthesis of soluble and insoluble proteins in lenses of rats kept for only six instead of 21 days on galactose diet and compared it with that of controls on normal diet. We also determined, the effect of oxidation by ferricyanide on the soluble proteins of lenses of

rabbits and calf, and made an attempt to characterize the abnormal protein which precipitates after oxidation by its solubility properties. The present report deals with these experiments.

## Experimental

### A. Material and analytical methods

In three out of the four experiments in the present series, the Sherman strain of rats, bred at Columbia University, was used. In one experiment, the rats came from the Rockland Farms. All animals used in every single experiment were born on the same day, but were not litter mates. Every experimental and control group contained the same number of rats and the average weight of the animals in the experimental and corresponding control groups was almost identical. At six weeks of age this weight was in the Sherman strain 95 to 105 gm. and in the Rockland Farms group 120 gm. The killing of the animals and the extraction and preparation of the lens were carried out in the way described in a preceding paper.[1]

The nitrogen determinations were carried out by microkjerdahl, glutathione determinations by the glucose-tryptophan-$H_2SO_4$ method, previously described.[2]

### B. Results

1. *Inhibition of the net synthesis of soluble lens proteins during the first six days of galactose feeding*

Four experiments of this type were carried out. In each experiment two groups of five- or six-week old rats, consisting each of eight to 12 animals, were kept on galactose diet for a period of six days, while two other groups of the same number of animals were kept on normal diet. On the day on which the experiments started, two groups of animals born on the same day as the experimental animals and controls, each group consisting of eight to 12 animals, were killed, and their lenses removed. The lenses of each group were separately homogenized under cooling in an ice water bath, and the insoluble albuminoid was removed by high speed centrifugation.

The supernatant was kept overnight frozen at −15°C. and the next day 0.5 cc. of the supernatant was precipitated with two cc. of a seven-percent TCA. The precipitate was washed with five-percent TCA and dissolved in 10 cc. of 0.5 NaOH. This solution was used for determination of the nitrogen, the supernatant of the TCA precipitation for the determination of glutathione and substances reducing ferricyanide at pH 7.5 at 37°C. The lenses from the experimental animals and controls killed six days later were treated in exactly the same way. In this way duplicate values for the soluble proteins of the lenses of animals killed at the beginning of the experiment, of animals on galactose diet and of controls on normal diet were obtained. Each value corresponded to one group of between eight to 12 animals.

This procedure made it possible to evaluate the variations in the amount of soluble proteins between various groups of animals of the same age and to compare it with differences between the galactose-fed animals and their controls.

The insoluble albuminoid was first washed with saline and then twice with distilled water, and dissolved in 10 cc. of half N NaOH. The results of these determinations in four experiments are listed in Table 1. In the first three experiments animals of the Sherman strain were used, which at six weeks of age weighed on the average between 95 and 105 gm. In experiment IV, the animals were obtained commercially from Rockland Farms and weighed 120 gm. on the average. As can be seen from Table 1, the differences in the amount of soluble protein between the two corresponding groups of animals killed at the beginning of the feeding period and of the control animals on normal diet varied between 0 and 5.5 percent, and those between the two groups on galactose diet varied between 3.0 and 7.0 percent.

TABLE 1

PROTEIN SYNTHESIS IN LENSES OF RATS FED ON NORMAL AND GALACTOSE DIET

| Experiment No. | No. Lenses | Days since Start of Experiment | Age at Time 0 | Diet | Wet Weight in mg./lens | Soluble Proteins in mg./lens | Insoluble Proteins in mg./lens |
|---|---|---|---|---|---|---|---|
| I | 32 | 0 | 42 | | 21.35 ±0.15 | 6.35 ±0.04 | |
| | 32 | 6 | | Normal | 23.6 ±0.4 | 8.15 ±0.00 | |
| | 32 | 6 | | Galactose | 22.8 ±0.4 | 7.1 ±0.1 | |
| II | 36 | 0 | 42 | | 19.5 ±1.00 | 6.98 ±0.2 | |
| | 34 | 6 | | Normal | 25.2 ±0.8 | 8.58 ±0.05 | 1.64 ±0.06 |
| | 36 | 6 | | Galactose | 23.5 ±0.2 | 7.02 ±0.18 | 1.52 ±0.05 |
| III | 46 | 0 | 35 | | 17.9 ±0.1 | 5.74 ±0.14 | |
| | 42 | 6 | | Normal | 21.6 ±0.2 | 6.72 ±0.02 | |
| | 42 | 6 | | Galactose | 20.9 ±0.2 | 5.75 ±0.2 | |
| IV | 48 | 6 | 42 | Normal | 25.7 ±0 | 8.4 ±0.14 | 1.9 ±0 |
| | 48 | 6 | | Galactose | 25.3 ±0.2 | 7.83 ±0.2 | 1.88 ±0.01 |

The amount of protein formed during the six-day period on normal diet, on the other hand, varied in experiments I to III between 17 and 28 percent of the values found at the beginning of the experiment. The amounts of soluble proteins in lenses of galactose fed animals calculated as average of two corresponding groups in every experiment, were in experiments II and III identical with those of lenses removed at the beginning of the experiment. It was higher by only 12 percent in experiment I.

These data indicate, therefore, that in two out of the three experiments a complete inhibition of the net synthesis of proteins in lenses of galactose fed animals took place, while in the experiment I the synthesis appears to be inhibited by about 60 percent. The wet weight of lenses, on the other hand, in the galactose fed animals increases during the experimental period, although the increase remained somewhat smaller than that found in animals on normal diet. The concentration, therefore, of soluble proteins in the lenses of galactose fed animals was lower in all experiments than that in animals on normal diet. In sharp contrast to this behavior of the soluble proteins is the behavior of insoluble albuminoid, which in experiments II and IV, in which it was determined, showed no significant difference between the experimental animals and their controls, although a significant increase in its amount in the six day period took place in both cases.

*2. Oxidation of soluble lens proteins by ferricyanide and its effect on their solubility*

The supernatant of lens homogenates of experiments I to IV, which was not used for the determination of nitrogen, was subjected to oxidation by ferricyanide. To this end, two samples of 1.0 cc. of a homogenate from every group of animals was mixed with 2.0 cc. of 1 M of phosphate buffer of pH 7.4, 3.0 cc. of a M/400 solution of $K_3Fe(CN)_6$, and 1.0 cc. of water, and the mixture was incubated in a water bath of 37°C. With every pair of samples was run one control, in which the $K_3Fe(CN)_6$ solution was replaced by the same amount of water.

After an incubation of 150 minutes, the optical density of the solutions were measured at 480 mμ on all samples. In samples from experiment I, one interim measurement was made also after 90 minutes. The absorption of these samples at this wavelength is due only to the turbidity of the solution, as neither $K_3Fe(CN)_6$ nor the product of its reduction, $K_4Fe(CN)_6$, show any significant absorption at these wave lengths. $D_{480}$ of the samples, therefore, was taken as a measure of the precipitation of

TABLE 2

REDUCTION OF $K_3Fe(CN)_6$ BY SOLUBLE LENS PROTEINS OF RATS ON NORMAL AND GALACTOSE DIET AND ITS EFFECT ON THEIR SOLUBILITY

| Experiment No. | No. of Lenses | Days since Start of Experiment | Age at Time 0 in Days | Diet | Time of Incubation | $K_3Fe(CN)_6$ Reduced in uM/mg. | $D_{480} \times 1000$ of Solution After Incubation | Protein Precipitated by Oxidation in % of Total Soluble |
|---|---|---|---|---|---|---|---|---|
| I | 32 | 0 | 42 | | 1½ hr. | | 212 ± 44 | |
| | 32 | 6 | | Galactose | | | 1030 ± 43 | |
| | 32 | 6 | | Normal | 2½ hr. | 104 | 684 ± 64 | |
| | 32 | 6 | | Galactose | | 137.5 | 1.300 ± 33 | |
| II | 36 | 0 | 42 | | 2½ hr. | 123 ±1.0 | 1060 ± 20 | 24.3 ±0.9 |
| | 34 | 6 | | Normal | | 113.5 ±4.5 | 830 ±130 | 21.7 ±2.4 |
| | 36 | 6 | | Galactose | | 156.4 ±2.3 | 1240 ± 21 | 40.8 ±0.9 |
| III | 46 | 0 | 35 | | 2½ hr. | 133.9 ±0.6 | 985 ± 75 | |
| | 42 | 6 | | Normal | | 112.8 ±1.0 | 750 ± 34 | |
| | 42 | 6 | | Galactose | | 151.6 ±1.5 | 1285 ± 55 | |
| IV | 48 | 6 | 42 | Normal | 2½ hr. | 100.0 | 970.0 | 23.3 |
| | 48 | 6 | | Galactose | | 122.0 | 1320.0 | 39.5 |

protein due to oxidation.

For this purpose, an average was taken of the two duplicates of samples coming from the same homogenate of each group of lenses. The average of the corresponding two groups of lenses of animals killed at the beginning of the experiment, and the experimental animals and their controls were then calculated, and these averages are listed in column 7 of Table 2, as well as the deviations of the average values found in everyone of the two groups from averages of the two groups. As can be seen, these deviations amounted up to 15 percent of the observed average of the two groups. There was a slight but significant decrease in turbidity of the oxidized homogenate after six days in animals on normal diet. In animals on galactose diet, on the other hand, there was an increase between 23 and 30 percent against the values found at the beginning of the experiment, and the differences against the controls in the experimental animals kept for six more days on normal diet ranged between 50 and 90 percent.

At the end of the incubation period after the determination of $D_{480}$ was completed, the oxidized homogenates were transferred into centrifuge tubes and the precipitate centrifuged off during 35 minutes at 12,000 revolutions in the Spinco Ultra Centrifuge. The supernatant of this centrifugation was decanted and precipitated immediately with 1/20 of its volume of 100-percent TCA. A certain amount of distilled water was then pipetted into the spectrophotometric cells used for the measurement of $D_{480}$ and to the test tubes used for the incubation and small amounts of precipitate remaining in these vessels were rinsed out and combined with the main bulk of the precipitate in the centrifuge tubes. The precipitate was repeatedly washed with distilled water, then once with 5.0-percent TCA and dissolved in 0.5 N NaOH. This solution was used for the direct determination of the amount of protein precipitated by oxidation.

Such determinations were carried out in experiments II and IV, of Table 2. As can be seen they confirm the results obtained by the determination of light absorption at 480 mμ as the amount of protein precipitated after oxidation was 70 to 95 percent higher in lenses of rats on galactose diet than in the controls. The supernatant which was precipitated with TCA was left for two hours in the refrigerator, the precipitate was centrifuged off and the optical density of the

supernatant at 420 mμ and 480 mμ was determined in all samples, as well as in a standard solution of $K_3Fe(CN)_6$, which was prepared by mixing the same amount of the reagent which was added to each sample with a corresponding amount of distilled water and TCA to a volume identical with that of the incubated samples.

The amount of ferricyanide which was reduced by the homogenate was then calculated by the difference in the optical density of the incubated samples and of the standard. The duplicate values from every single homogenate obtained from one group of animals did not differ more than two percent from each other. The differences between the average values from two corresponding groups of lenses differed maximally four percent from each other. The reduction of $K_3Fe(CN)_6$ by the homogenate was partly due to the presence of glutathione and ascorbic acid in the homogenate. The part of the latter in the reduction could be disregarded, but not that of the first one.

Determinations of the glutathione on TCA filtrate of the homogenate showed that after six days of galactose feeding the concentration of this compound dropped to such a small fraction of the initial value that it could not be determined any more with sufficient precision. For the comparison of the reduction of $K_3Fe(CN)_6$ by the soluble proteins in galactose fed rats and controls, the first was assumed to be identical with the total reduction of the homogenate and the second was calculated by subtracting from the total reduction, the reduction due to the difference between the concentration of glutathione in controls and experimental lenses.

As can be seen from Table 1, the amount of ferricyanide reduced by the weight unit protein decreases slightly in the rats on normal diet during the six day period. The decline varied between eight and 15 percent of the value at the beginning of the experiment. In sharp contrast with that, the reduction of the ferricyanide by the soluble lens proteins of rats on galactose diet went up between 19 and 27 percent. The difference between the reducing power of the soluble proteins of galactose-fed rats and those on normal diet, therefore, was still more pronounced, varying between 33 and 39 percent in experiments I, II, and III. It was 19 percent in experiment IV.

### 3. *Fractionation of the lens proteins from lenses of rats on normal and galactose diet*

To interpret the marked changes in the reducing power of the soluble lens proteins under the influence of galactose feeding, the increase in the protein which precipitates after oxidation, it appeared first of all necessary to examine whether these phenomena are not due simply to a shift in the ratio of α and β crystalline fractions of the soluble proteins, as the latter fraction has a much higher reducing power than the first one. The fractionation was carried out according to a modification of the method of Burky and Woods[4] on one part of the homogenate in experiment II. The supernatant of the lens homogenate was adjusted to pH 5.2, and the resulting precipitate, after standing for a few hours at 4°C., separated by centrifugation. A measured volume of the supernatant was then dialyzed at 4°C. against often changed distilled water. A second precipitate appeared, which has been previously shown in our laboratory by electrophoretic mobility and the cysteine determination not to differe significantly from the first α crystalline precipitate. The pH of the outside and inside fluids remained at 5.0 during the whole period of dialysis.

The content of the dialysis bag was centrifuged, the supernatant decanted, the precipitate washed repeatedly with distilled water, and the washings combined with the supernatant. The supernatant then was filled up to a certain volume and its nitrogen determined. Although the supernatant contained still a small contamination of α crystalline which could be demonstrated by continuous flow electrophoresis, it consisted mainly of β crystalline and the ratio, therefore, of the

content in proteins between the supernatant and the total soluble fraction from which it was derived, could be assumed to very nearly correspond to that of β crystalline to total soluble protein.

The determination in experiment II of this ratio on lens homogenates from rats on normal and galactose diet showed no significant differences between these two groups of rats, the ratio being 65 percent in the first and 67 percent in the second case.

4. *The effect of oxidation of α and β crystalline*

As a first step in the investigation of the nature of the precipitable protein, the effect of oxidation by ferricyanide on the separated β *and* α crystalline fractions was determined. To this end, the two α crystalline precipitates, washed free of β crystalline, were dissolved in a diluted $NaHCO_3$ solution which was then adjusted to pH 7.4. The oxidation experiments then on the two fractions were carried out exactly like those on the total soluble protein fraction. The concentration of the protein in solution in the two fractions corresponded to their concentration in the lens homogenate. Furthermore, the two fractions were combined and all the reagents were added from more concentrated solutions in such a way that the final concentration of β crystalline in the mixture was the same as in the sample containing only one of the two proteins. While the α crystalline did not show any turbidity after an incubation of 2½ hours, a heavy precipitate appeared already after 30 minutes in the samples containing the β crystalline fraction. $D_{480}$ of the solution containing only the β crystalline was 1.526 after two and one-half hours. The protein precipitate after oxidation, therefore, forms part of the β crystalline fraction.

The determination of the total amount of the protein precipitated after two and one-half hours in this fraction showed that up to 73 percent of the protein was precipitated under these conditions. When the α and β crystalline fractions were recombined, the precipitation after oxidation amounted to 57 per cent of the β crystalline present. The presence of α crystalline, therefore, in solution seems to exert a somewhat retarding influence on the precipitation of the oxidized β crystalline. Preparations of β crystalline from lenses of one year old rats did not show any significant precipitation after oxidation.

5. *Oxidation of soluble lens proteins and β crystalline of rabbit and cattle*

To test whether a protein which precipitates after oxidation is present in lenses of young animals of other species, the same type of experiments were carried out on homogenates from lenses of two-week and six-month-old rabbits, and of four-month-old calves. The total soluble protein in both these species did not show any significant precipitation after two and one-half hours of incubation with ferricyanide. However, when β crystalline fractions were prepared in the same way as was done in the case of rat lenses, the β crystalline from two week old rabbits showed, after two and one-half hours, a heavy precipitate, and $D_{480}$ of the turbid sample was 0.760. No such precipitate was observed with the β crystalline from the six-month-old rabbit, nor the four-month-old calf, although the protein solutions were adjusted to the same concentration of protein. The lack of precipitation in the total homogenate in the two-week-old rabbit may be due either to the inhibiting effect of α crystalline, or some other constituent of the lens, which was removed during the preparation of β crystalline.

## Discussion

Our experiments indicate that the inhibition of the net synthesis of soluble proteins coincides more or less completely with the beginning of the galactose feeding. This suggests, first of all, that this effect is a direct effect of galactose on the lens and not a secondary effect due to damage to

other tissues produced by the galactose diet. In the latter case, we would expect rather a slower start and continuing increase in the inhibition, as the damaging effect of galactose should become more pronounced with the continuation of the galactose feeding.

This assumption about the mechanism of action of galactose appears consistent with the important findings of Schwarz and Goldberg.[5] These authors found an accumulation of galactose-1-phosphate in the lens capsule of rats kept on galactose diet. They also found that in infants affected by hereditary galactosemia, this ester also accumulates in the erythrocytes, and this accumulation is accompanied by a decrease in the respiratory mechanism of the red cells.

It is reasonable, therefore, to assume that the accumulation of galactose-1-phosphate in the epithelium of the lens will lead to an inhibition of the oxidative metabolism of this epithelium. This oxidative metabolism obviously is necessary for protein synthesis and its inhibition, therefore, would induce an inhibition of the protein synthesis in growing rat lenses.

Although our findings do not permit conclusively to decide whether the stoppage of the net synthesis of soluble lens proteins is due to an inhibition of the synthesis of the synthetic processes themselves, or to an increase in their breakdown, they seem to support rather the first alternative for the following reasons:

The data in Table 1 concerning the effect of the galactose feeding on the wet weight of the lenses indicate that the increase in wet weight is only somewhat retarded, although the net protein synthesis is practically completely inhibited. The growth in wet weight, therefore, in lenses of galactose fed animals is due almost exclusively to the uptake of water. This swelling certainly will result in more or less marked disruption of the physical continuity of the content of the lens fibers and, therefore, by analogy with the influence of such structural disruption on catabolic processes, should favor an increase in protein breakdown. This effect on the breakdown could be expected to be intensified with the intensification of the structural changes with continuation of the galactose feeding. If the direct synthesis of lens protein, therefore, would not be affected, the effect of the galactose feeding in the first six days should be less pronounced than in the subsequent two weeks.

The comparison of our present series of experiments with that previously reported on animals kept for 21 days on the galactose diet shows, however, that the effect on the net synthesis of soluble proteins is a maximal one already during the first ten days. The predominant role of proteolysis appears, furthermore, improbable on the basis of the important observations of Fromageot and Prioux,[6] who reported that the presence of strongly reducing substances like cysteine and ascorbic acid is necessary for a significant activity of proteases in rat lenses. Our findings that glutathione largely disappears from the lenses of galactose fed rats after six days suggest, therefore, that the activity of the lens proteases would rather decrease than increase in lenses of galactose fed rats.

Our experiments also demonstrate that changes in the chemistry of the soluble proteins themselves appear very rapidly after the start of the galactose feeding, and lead to an accumulation of a specific protein fraction at a level which does not seem to increase significantly afterwards. This can be inferred from a comparison of the data on the increase in the turbidity after oxidation of the soluble proteins in lenses of galactose-fed animals in the present series of experiments with that in experiments with a more prolonged experimental period.

A striking increase in the reducing capacity of the soluble proteins under the influence of galactose feeding, which cannot be only due to an increase in β crystalline, or an increase in the total content in cysteine of soluble lens proteins, indicates changes in the physico-chemical properties of the β crystalline fraction, which could result in a greater

availability of SH groups of this protein for the oxidizing agent. The decrease in the reducing power of the soluble proteins and the precipitable protein with increasing age which parallels the decrease in the rate of synthesis of soluble proteins of the lens seems to support the assumption that the protein which is precipitated after oxidation represents an intermediate stage in the differentiation of lens proteins.

The increased accumulation of this intermediate protein during galactose feeding suggests that the experimental diet stabilizes the soluble lens protein at an intermediate stage of their development, and inhibits their transformation into a final form. Such an accumulation of an intermediate in a chain of reversible reactions will finally result in complete inhibition of the whole reaction chain.

Our failure to obtain evidence for the presence of such a precipitable protein in homogenates of calf lenses cannot be regarded as a valid objection against this concept. The decrease in the amount of the precipitable protein with progressing age of the rat and its disappearance at the time when the growth curve of the lens flattens out, indicates a parallelism between the rate of synthesis per unit weight of soluble lens protein and the amount of the precipitable protein. This rate is probably much lower in the lenses of calves of three to four months, which we investigated, than in those of rats between five and 10 weeks of age. This explains also why an increase in the turbidity of β crystalline after oxidation is found in rabbits only at a very early age, namely, two weeks after birth. The rate of synthesis per unit weight in the rabbit lenses of later age is probably also much smaller than in the rat lenses.

The presence in the lens of a protein fraction which is much more predominant in lenses of embryos and young animals than in that of older animals recently has been also demonstrated by electrophoretic methods in two different laboratries. François and Rabaey[7] found such a fraction with a very low mobility in paper electrophoresis, which in embryos and in young animals represents almost one third of the total protein of the lens and is also present at this level in the nucleus of older animals, but is almost non-exisent in the cortex of these animals formed later. Fuchs and Kleifeld[8] reported almost identical findings on lenses of guinea pigs. As François and co-workers previously found[9] even four different fractions in paper electropherograms of lens proteins of rats, it seems possible that this slow moving protein, which is referred to by François and Rabaey as embryonic protein, may be identical or related to the precipitable protein found in rat and rabbit lenses. This problem is now under investigation in our laboratory.

## Summary

1. The net synthesis of soluble protein in lenses of five- to six-week-old rats kept on a standard galactose diet for six days is inhibited 60 to 100 percent.

2. During the six-day feeding period, there takes place in the lenses of galactose fed rats a strong accumulation of a protein which, after oxidation with ferricyanide, becomes insoluble and precipitates.

3. These proteins form part of the beta crystalline fraction of the soluble lens proteins.

4. The synthesis of insoluble proteins proceeds normally in galactose fed rats.

5. The beta crystalline from two-week-old rabbits contains a protein fraction which precipitates after oxidation by ferricyanide.

6. This fraction is not present in demonstrable amounts in lenses of six-months-old rabbits, and from four-month-old calves.

*630 West 168th Street (32).*

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## Discussion

Dr. John W. Patterson (Cleveland): Dr. Dische and his group are to be congratulated for the progress they are making in the difficult field of lens protein metabolism.

In simplest terms, we know that the transparency of the lens is maintained in health, and that when there is alteration of normal physiology cataracts may result. More specifically, we now know that maintaining transparency is a dynamic phenomenon requiring oxygen, energy-yielding substrates, and essential synthetic substances. A deficiency of any of these will lead to cataracts.

These initial ingredients then interact and interrelate in specific ways to form a chain or network of specific links that must be maintained. Interruption of certain of these links may result in the formation of cataracts.

Finally, there must be an equilibrium with respect to water balance, electrolyte balance and protein balance if the proper physiochemical properties and relationships for transparency are to be maintained. In the case of galactose cataracts, the chain of events is becoming more evident. Recent work by Kalckor and his group has shown that galactosemia is a hereditary disease in which there is a deficiency of galactose-1-phosphate, uridyl transferase. This enzyme is located largely in the liver, and a deficiency blocks the metabolism of galactose and galactose-1-phosphate with a subsequent increase in the blood level of galactose.

Galactose cataracts are secondary to this galactosemia, inasmuch as they occur only when there is a high level of galactose in the blood following the saturation of the enzyme system, and can be prevented by lowering the blood galactose level.

It has been shown in our laboratory that the development of these cataracts in rats is dependent upon the amount of galactose reaching the eye. Nutritional studies which we have been conducting for several years indicate that excessive amounts of galactose interfere with glucose metabolism. This is based on the observation that galactose cataracts can be delayed or prevented by providing other substrates as sources of lens energy.

Thus, under ordinary circumstances galactose blocks the production of energy that is needed for maintaining protein synthesis, as has been indicated in Dr. Dische's paper. The exact links between energy production and protein synthesis and between protein imbalance and the failure to maintain transparency still remain to be clarified.

Furthermore, the various steps in the protein synthesis must also be elucidated.

This is a difficult field in which techniques are still being developed, and we certainly wish Dr. Dische success as he contines his investigations.

I would like to ask Dr. Dische if he has any evidence with regard to the nature of the precipitable protein fraction. I would also like to ask Dr. Dische if he has any evidence with regard to protein or glutathione changes in other types of cataracts.

Dr. Zacharias Dische (closing): As far as the first question is concerned, we believe from our experiments that this precipitable protein is an intermediate stage in the development of the lens proteins, and that its accumulation in the galactose cataract is due to the fact that the chain of reactions that lead to the final form of the lens proteins is interrupted at the later stage. This leads to an accumulation at a preceding stage which is represented by the precipitable protein, and then to complete stoppage of the synthesis by the mass action.

We have also some evidence, which I could not present here due to lack of time but which is in the paper, that the precipitable protein is lower in certain amino acids than the beta crystalline, from which it is derived. The beta crystalline is a mixture of several proteins, and it is possible that one of these proteins is precipitated after oxidation; but it is also possible that, as Dr. Kinoshita pointed out, there is a hydrogen bond split due to the ferricyanide oxidation, and that one part of the beta crystalline is split off which contains less of certain amino acids.

As far as the second question is concerned, we are now engaged in our laboratory in investigations of a similar type on radiation and tryptophan deficiency cataract; and although we have no definite results, we can say that the picture is very differ-

ent in either of these cataracts. The radiation cataract differs also from the tryptophan deficiency cataract.

One of the things we found that was rather intriguing was that in irradiated rat lenses at the stage where there are already small but distinctly visible cataractous changes, there is only a very small change in the glutathione concentration. This seems to be at variance with the findings of Pirie and Heyningen in rabbits.

We give rats the same doses at which they irradiated their rabbits, and we would expect a stronger effect on the glutathione. We could not find it. After six days of feeding galactose, there is almost no glutathione in the lenses, but there is plenty of glutathione in rat lenses 12 weeks after irradiation with 2,000 or 3,000 r.

## INSULIN AND THE LENS*

TIBOR G. FARKAS,† A.B.
*Cleveland, Ohio*

AND

JOHN W. PATTERSON, M.D.
*Vancouver, British Columbia*

Experimental animals in which the insulin producing cells have been destroyed by the injection of alloxan develop cataracts some weeks after the injection of the diabetogenic agent.[1] Such cataracts can be prevented by feeding a diet containing substrates that do not require insulin for utilization.[2] This suggests that cataracts are formed when there is a deficiency of energy yielding nutrients for the lens.[3] Since the lens depends on glucose for a major portion of its energy and since the level of glucose in diabetes is high, there apparently is an impairment in the ability of the lens to metabilize glucose in the absence of insulin. This is supported by the fact that the injection of insulin prevents the development of cataracts in alloxan diabetic rats.[4] It is the purpose of this paper to describe some experiments on isolated rat lenses, with respect to the effect of insulin on the glucose uptake.

When the lens is removed from the eye of a rat and placed in an incubation medium consisting of Tyrode's solution containing glucose, the glucose is removed from the medium at a constant rate for a period of several hours. The details of this procedure have been described.[5] The rate at which glucose is removed from the incubation medium by a rat lens is dependent upon the concentration of glucose in the medium (fig. 1). The uptake of glucose is maximal at approximately 200 mg. percent concentration of glucose. At higher levels, the uptake is considerably less. The reasons for the decreased uptake at the higher concentration are not known.

The effect of an insulin deficiency can be

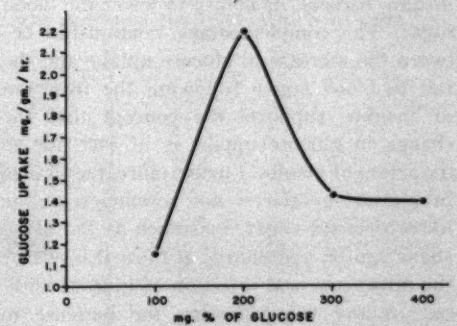


Fig. 1 (Farkas and Patterson). The rate at which glucose is removed from the incubation medium by a rat lens is dependent upon the concentration of glucose in the medium.

* We wish to express our appreciation for the support received from the National Institute of Arthritis and Metabolic Diseases, of the National Institute of Health, U. S. Public Health Service, and for a fellowship from the National Council to Combat Blindness, New York City in support of one of the authors (T.G.F.). We are indebted to Mr. Robert F. Ivory for skillful technical assistance.

† Fight for Sight Research Fellow.

demonstrated by studying lenses from rats with alloxan diabetes. When the uptake of glucose in lenses obtained from diabetic rats is compared with lenses obtained from normal rats, there is a rapid fall in the ability of the lens to take up glucose following the induction of diabetes.[5] A decrease to 45 percent of the normal level is observed within seven days after the induction of diabetes and represents one of the early metabolic changes in the diabetic lens. This change is not related to a change in blood sugar levels, inasmuch as the glucose uptake is not influenced by the marked changes in blood sugar level that can be produced by starving or feeding diabetic animals.

The effect of an insulin excess can be demonstrated by studying lenses from rats that have been injected with insulin. The injection of insulin into a rat one hour prior to sacrifice and removal of the lens significantly increases the uptake of glucose in lenses from normal animals as well as lenses from diabetic animals. This increase in the uptake of glucose is dependent on the length of time that lapses between the injection of insulin and the sacrifice of the animal to remove the lens (fig. 2).

The optimal time-interval for increasing glucose uptake following the injection of insulin corresponds with the optimal time for another manifestation of the action of insulin, namely, its ability to lower the blood sugar. The complementary relationship between the increase in glucose uptake and the fall in blood sugar following the injection of insulin, supports the concept that the change in glucose uptake is in fact due to the action of insulin. Furthermore, the change in glucose uptake is not secondary to the lowered blood sugar, inasmuch as the blood sugar can be maintained at normal levels by the intraperitoneal injection of glucose without in any way affecting the increase in glucose uptake that follows insulin injection.[5]

The increase of uptake following the injection of insulin is highly significant and

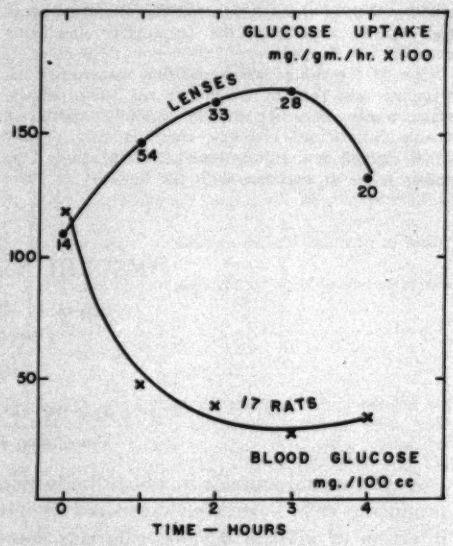


Fig. 2 (Farkas and Patterson). Effect of 20 u intravenous insulin on glucose uptake of lens and blood sugar of rats.

may be as much as 50 to 60 percent greater than that observed in nontreated controls. This contrasts with the failure of 0.5 units of insulin to produce an effect when added directly to the incubation medium, even though adequate time was allowed for it to have an effect (fig. 3). This observation is in agreement with that of Harris.[6,7]

The difference between the in vivo and in vitro insulin effect provides a clue to the mechanism of insulin action on the lens. Taken at face value, it suggests that insulin does not act directly on the lens. Conceivably insulin might affect lens metabolism only after it is modified in some other organ, or only in the presence of some other substance, or by the production of a mediating substance.

The role of other organs in the in vivo insulin effect can be investigated by studying the effect of injected insulin in animals from which various organs have been surgically removed. Following complete evisceration[8] below the level of the diaphragm, including nephrectomy, there is no demon-

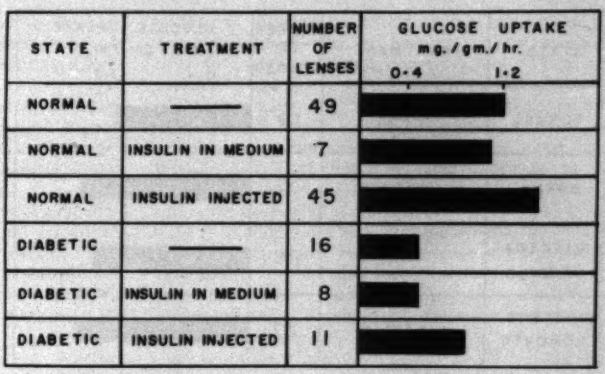


Fig. 3 (Farkas and Patterson). Effect of insulin on glucose uptake of rat lenses.

strable insulin effect. Lenses from sham-operated controls do show an insulin effect (fig. 4). In these preparations insulin is injected by way of the jugular vein and not through the tail vein, in order that one can be sure that the insulin is distributed throughout the arterial circulation.

The failure to achieve an insulin effect following evisceration suggests that one or more of the organs that were removed plays a role with respect to the action of insulin on the metabolism of the lens.

The role of the liver can be partially studied by removing all the viscera with the exception of the liver. In this preparation the blood supply to the liver from the gastrointestinal tract is interrupted but the blood supply from the hepatic artery is maintained. Under these circumstances it is possible to demonstrate an insulin effect. However it is also noted that the uptake of glucose without insulin is appreciably increased (fig. 5).

The first observation implicates the liver as an organ of importance in insulin metabolism. However, the second observation suggests that the problem is not as simple as finding a single organ, but that there may be an interaction between organs. Thus, possibly the removal of the viscera other than the liver also removes a physiologic inhibitor of the glucose uptake by the lens. One could speculate with regard to the interpretation of this isolated bit of data. However, it is probably wise to await the collection of additional information before theorizing too much. One could conclude, however, that the available data is consistent with the viewpoint that injected insulin affects an organ, perhaps the liver, which in turn has

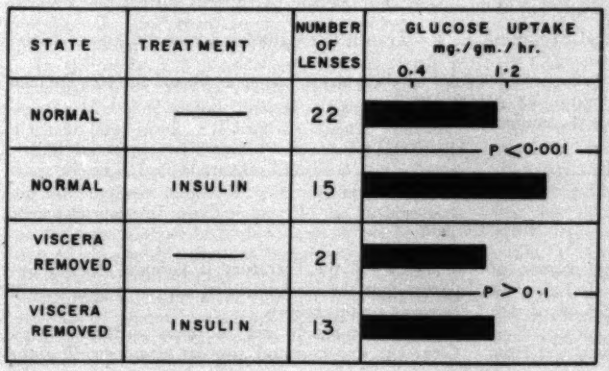


Fig. 4 (Farkas and Patterson). Effect of evisceration on glucose uptake of rat lenses.

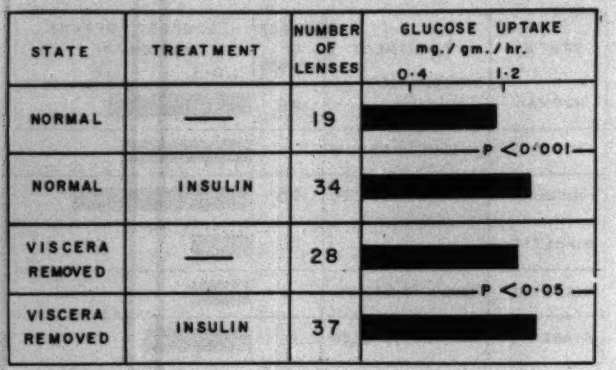


Fig. 5 (Farkas and Patterson). Effect of evisceration without hepatectomy on glucose uptake of rat lenses.

a marked influence on the glucose uptake of the lens.

## Summary

The glucose uptake of isolated lenses from animals with different types of conditions was studied. The glucose uptake was decreased significantly following the destruction of the insulin-producing cells by alloxan. The uptake was significantly increased following the injection of insulin in the rat prior to death. The magnitude of this effect corresponded with respect to time with the recognized capacity of insulin to lower the blood sugar. However, the change in glucose uptake was not secondary to the change in blood sugar. Insulin added to the incubation medium did not alter the glucose uptake of isolated lenses. This suggested that the insulin effect was mediated through some organ. This concept was supported by the facts that complete evisceration abolished the insulin effect, whereas evisceration without removal of the liver permitted the insulin effect to be demonstrated.

*2109 Adelbert Road (6).*
*University of British Columbia.*

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## Discussion

Dr. Jin H. Kinoshita: During the course of the discussion I would appreciate it if I may be permitted to use slides No. 3 and No. 4 of Dr. Patterson's presentation.

This is an extremely complex problem, and from a review of the literature it becomes readily apparent that the mechanism of action of insulin still remains quite obscure.

Insulin seems to act in many different ways. Clear-cut experimental support demonstrating the

fact that insulin influences many biochemical and physiochemical processes has been difficult to obtain, so that in many cases only suggestive evidence is available.

Perhaps the only action of this hormone which has received fairly consistent experimental support is that insulin in some way promotes the entry of sugars into the tissues. By what mechanism insulin acts in this case, is again not known.

This paper just presented is another which indicates that under certain experimental conditions the glucose uptake is facilitated by insulin. However, in this study with lens, it appears different, in that another organ apparently participates in initiating the insulin effect. I don't think this has been shown by other tissues.

I would like to ask Dr. Patterson whether there are any other organs which behave similarly to lens, in that it requires another organ to initiate the insulin effect. In this regard I have a comment on the time course curve of the insulin action.

It seems remarkable that the effect of insulin is so immediate as far as the lens glucose uptake is concerned; in fact, the response of the lens is as rapid as the lowering of the blood sugar. This is particularly interesting in view of the work of Reynold et al., who found that after injecting insulin the effect on blood sugar was immediate, but the insulin effect on the liver was observed much later.

The fact that the lens effect is immediate, and since the blood sugar curve is most likely not influenced by evisceration, suggests that the organ participating in this insulin-initiating action does not modify insulin but most likely supplies a particular substance.

Another interesting fact on this graph is the sharp drop of lens glucose uptake curve after three hours. I wonder what explanation the authors might offer in this respect.

One wonders what the curve would look like if another dose of insulin were given at this point where the sharp drop occurs, namely, at the three-hour mark.

Another aspect that is somewhat puzzling is that when insulin is injected in the diabetic, the glucose uptake does not return to the level observed with the normal lens.

In the diabetic I would have predicted that if insufficiency of insulin is the cause of the low uptake of the lens, the injection of insulin should have at least brought it back to normal lens level.

DR. ZACHARIAS DISCHE (New York): I should like to ask Dr. Patterson whether the glucose uptake in these experiments means only the total disappearance of glucose, or did he differentiate between the glucose breakdown and simple penetration of glucose into the lens from the environment?

DR. E. ALBERT ZELLER (Chicago): I would like to ask one question about the curve in which depression of glucose uptake was shown with increase of glucose concentration—the optimum of glucose uptake and optimum glucose concentration.

That is the type of action we find very often in enzyme systems—that we have an optimal reaction velocity and an optimal concentration. When we increase it we get a depression of the velocity.

Do you have any indication that this type of curve might relate to similar concentration relationships, as, for instance, between glucose and hexokinase? That might be some indication as to the initial step of the glucose uptake, which is still unknown as to what it consists of.

It is certainly quite disturbing to find a system like the lens where insulin does not react directly, in contrast to the classical experiments with rat diaphragm, where glucose alters or halts the preparation directly. Washing of the muscle does not remove the insulin.

Here an important point comes up: How long has glucose been in contact with the lens. It might be that there is a time factor. It may take more time for lens than other tissue to interact with insulin. After all, the other well-studied organs with which insulin reacts, such as muscle or liver, are organs coming from different parts. Here in the lens we have an ectodermal tissue, and it might be that this is a reason why we see quite a difference in response rates.

DR. JOHN E. HARRIS (Portland, Oregon): We have done some experiments which bear on the last question. We agree, in another species at least, the rabbit, with the results that Dr. Patterson has presented, namely, that in the diabetic rabbit lens we find a marked decrease in glucose uptake or glucose utilization by the lens.

In that system, however, if one measures the turnover of glucose, using glucose uniformly labelled carbon-14, one finds that that lens is turning over glucose more rapidly than is the normal lens. Therefore, I would not anticipate that this turnover rate is not appreciably altered by insulin, and therefore I would not anticipate that the glucose would be of much significance.

There is one thought that I am sure Dr. Patterson has in the back of his mind, and that is that insulin is present in the liver. We don't know the final molecular weight of insulin as it is active in the body. It may not be 0.036. If that were the case you would not expect it to get in the anterior chamber.

Some years ago we did some experiments using $I^{131}$ labelled insulin injected into the rabbit, and we were able to demonstrate some $I^{131}$ activity in the supernatant of aqueous precipitated with 10-percent trichloracetic acid. However, in the coagulum we found no activity, and therefore we concluded that we had lost all our activity probably; we thought perhaps we lost the $I^{131}$. Actually, I am not sure that is the case, and we are investigating these experiments at the present time.

DR. V. EVERETT KINSEY (Detroit): Dr. Dische put his finger on one of the difficult points of the problem.

Dr. Patterson's results correlating glucose utilization with blood sugar level are quite convincing. Dr. Kuck and I have been able to demonstrate that if

a lens is placed in solutions containing various amounts of glucose, the concentration of glucose rises. If the glucose consumption of these lenses is then determined using the Patterson-Farkas technique, one finds an apparent decreased consumption.

The explanation, I think, is that the glucose comes out of the lens, adding to that in the Tyrode's solution, not that there is any difference in glucose consumption. We have obtained similar results with lenses removed from diabetic rats and again we believe that this indicates only that these lenses contained extra glucose to begin with. Since the consumption is calculated from the difference in concentration in the Tyrode's solution before and after incubation, the decreased consumption is only apparent.

DR. JOHN W. PATTERSON (closing): I would like to thank those who have raised questions with regard to our presentation. It indicates the interest in this particular field. I shall try to answer the questions as briefly as possible.

The first question had to do with the effect of other organs as far as the action of insulin is concerned.

There are two points that might be raised in answering this question. If one recalls the curves that were published a number of years ago by Soskin and Levine, relating the glucose utilization in the whole animal, the dog, to the blood sugar level, one got a somewhat sigmoid-shaped curve. What we did not do was to extrapolate that curve to zero blood sugar level. If we had done that it would have to come back to the origin.

The curve then becomes a diphasic curve. If this is done, also with their curve as determined in diabetic animals, the suggestion is that the two humps that contribute to this diphasic curve may behave differently in diabetes than they do in the normal, suggesting that in the animal as a whole there are different types of tissues which respond differently to lack of insulin; and probably a corollary to this would be that they probably respond differently to insulin.

The second point: Soskin and Levine have also done another experiment in which they measured the glucose uptake on the basis of arteriovenous differences in the extremity. These experiments were done in animals that had the blood sugar maintained by intravenous drip, and in whom insulin was present in maximal amounts.

With this system they compared animals with the liver removed and animals in which the liver was intact, and under these circumstances they found that the uptake of glucose in the extremity was much better in the presence of the liver as compared with the preparation in which the liver was absent. So, there are suggestions in the literature regarding the effect of other organs as related to insulin action.

The next question raised related to the rapidity of the effect of insulin on the glucose uptake. I don't think there is any particular answer to this. Another paper was referred to in which the effect was much slower as compared with the glucose change; if the glucose uptake were secondary to this phenomenon referred to which is slower, then it should also be slower.

If, on the other hand, it is independent of that, then we have no way of predicting how fast the effect should be on glucose uptake. The facts are as presented, and we must make our theories fit with the facts in whatever way they happen to come out.

The next point raised was related to the injection of insulin in diabetic animals, and as to why it did not come up to normal levels. I would like to point out that this experiment was done with a limited amount of insulin acting for a limited amount of time. If insulin is allowed to act for a longer period of time, one would expect it to come up to normal levels.

The question was also raised as to the sharp drop-off in uptake at the time of four hours after the injection of insulin as related to the three-hour level. Again there is no specific answer to that until further work is done which may explain it.

The question was asked as to what the effect of the second dose of insulin might have if given at this particular point. We have done one experiment in which smaller amounts of insulin were used, where the effect was much less marked, and then we compared that with injecting the same amount of insulin in two divided doses. Under these circumstances one gets a somewhat accumulative effect with two divided doses.

Dr. Dische asked a question as to what we were measuring—whether it was glucose disappearance or glucose uptake as measured in the lens. We are measuring glucose disappearance from the medium.

Dr. Harris has studied this point and has come to the conclusion that under normal circumstances the rate of glucose disappearance is determined by the rate of glucose utilization. This is not, however, under the circumstances that Dr. Kinsey referred to, wherein the lens is transferred from the medium of high glucose concentration to a medium with low glucose concentration.

The question was raised as to the length of time insulin is in contact with the lens in vitro, and whether there had been a long enough period of contact for the insulin to have effect. In reply to that question, I might say that one can have insulin in contact for as long as two hours in vitro with no effect, whereas one can demonstrate a very significant effect in one hour of contact when injected within the animal. So, there is still a discrepancy as relating to that point.

I would like to thank Dr. Harris for his comments and suggestions regarding the possibility that insulin may be modified. I certainly have no explanation. Until further work is done, I don't think anyone will have an explanation for the discrepancy between glucose uptake (which we agree to) versus the glucose turnover. Besides the glucose turnover rate, one would have to know whether the radioactive material in such a turnover is actually glucose or some metabolic product of glucose metabolism.

# THE PROTEIN COMPOSITION OF THE HUMAN LENS*

J. François, M.D., and M. Rabaey,† M.D.
*Ghent, Belgium*

Data on the protein composition of the human ocular lens have improved considerably since the development of modern techniques of electrophoresis. Mörner and Krause's old, simple schema, according to which the soluble proteins of the ocular lens included α-crystallin, β-crystallin and sometimes a small quantity of γ-crystallin, has become untenable and requires complete revision.

With the improvement in the techniques of protein separation it became clear that the problem of the proteins of the ocular lens is more complex than was initially believed, even after the first results of paper electrophoresis. In the course of our investigations we realized again and again that the results obtained by a certain technique are only relative results as against the increasingly complex schema of the protein composition of the ocular lens. Yet these results, although incomplete, can be regarded as having afforded important information (1953, 1954, 1955, 1956 and 1957).

So we have worked out a new method of fractionation of lens proteins (1955) and an immuno-chemical analysis of extracts of bovine lens by double diffusion in agar-gel and electrophoresis (1956); these experiments show there exist at least eight different antigens; only the fraction I (α-crystallin) possesses organ specificity; whereas, the other fractions possess species specificity.

Other investigations (1957) have established that there exists, in the bovine lens, a third important water-soluble protein fraction, distinct from other proteins and representing an embryonic protein. Indeed:

1. During embryonic life fraction III appears at a very high concentration, namely 18 to 24 percent, of water-soluble protein in bovine embryos of a length less then 7.0 cm. and more than 30 percent in embryos longer than 7.0 cm.

2. At least in embryos larger than 7.0 cm., during the embryonic period up to birth, the concentration of the various fractions remains remarkably constant; the three protein fractions increase simultaneously in a linear fashion.

3. After birth the situation changes. While fractions I and II continue to increase progressively, the concentration of fraction III diminishes significantly so that the absolute quantity of it in the lens increases very little after birth and not at all after the age of six months.

It could be argued that this lack of increase of the soluble fraction III is due to the freeing of part of it as an insoluble albuminoid. In embryos the amount of albuminoid is very small (three to five percent), but it also remains small for some time after birth. At the age of one year, soluble protein accounts for 96 percent, at three years for 92 percent, and at four years for 87 percent of the lens total. From that point on, the percentage of albuminoid rises markedly. But also from that point on, it can be seen that fraction I (α-crystallin) diminishes, while fraction II increases linearly, and fraction III remains practically unchanged. These facts allow the conclusion that the percentage of an albuminoid rises to a large extent by the denaturization of the α-crystallin in the nuclear part of the lens.

4. These facts are further substantiated by comparing the cortical and nuclear part of the lens. During embryonic life the two parts differ only slightly in the percentages of their various protein fractions. After birth, however, only a minute quantity of fraction III is synthesized in the cortical region.

* From the Ophtalmologie Clinic, University of Ghent (Director: Prof. J. François).
† Research fellow of the National Funds for Scientific Research.

We may thus conclude that fraction III is not a denaturization product of the aging α-crystallin, but a distinct protein which is synthesized, in the bovine lens at least, during embryonic life only.

At birth the lens of the calf already contains two thirds of the amount of fraction III found at 10 years of age, while in this period the total quantity of proteins increases fourfold. The lens of a six-month-old calf contains as much embryonic protein as the lens of a 10-year-old, while the total amount of protein is doubled during the same period.

It is impossible in this paper to present a detailed report on all the data which we have obtained. We must confine ourselves to a discussion of a few results obtained during an investigation of the protein composition of the normal human ocular lens.

It should first of all be pointed out that data on free electrophoresis of the proteins of the human ocular lens are unknown to us.

As we described in 1953, the protein extract from the human ocular lens can be separated, by means of paper electrophoresis, into four different components. The technique used for this purpose was that of Grassmann and Hannig (apparatus Elphor H, veronal acetate buffer pH 8.6, $\mu = 0.1$).

This paper electrophoretic separation of four fractions deviates to a considerable extent from the results obtained in the majority of mammals (cow, horse, hog, rabbit, and some 20 other species), in which the same technique revealed a general schema of three different protein components among which the one showing the swiftest migration corresponds with what was formerly known as α-crystallin.

Only certain primates had a paper electrophoretic lenticular protein pattern which resembled that seen in man (for example, the pattern in the lenticular extract from a young gorilla [Gorilla gorilla L.] is indistinguishable from that in human lenticular extract).

A detailed study of the electrophoretic pattern of the lenticular proteins in man shows the following fractionation of the four protein components (fig. 1):

The swiftest migrating fraction shows the highest concentration. This fraction closely corresponds, as to mobility and other properties, to the swiftest migrating mammalian fractions, which was characterized as α-crystallin. In addition there are three fractions close together and in about equally high concentration. Since these fractions are so close together, separation for quantitative reading is sometimes very difficult, and relative errors in the values obtained are more important. In order to insure comparable results the largest possible number of electrophoretic patterns were obtained with the same extract, and the average value of the various readings was taken.

Although it deviates from the mammalian, this schema still seems relatively simple. Difficulties arise, however, when investigations are extended to lenses from subjects

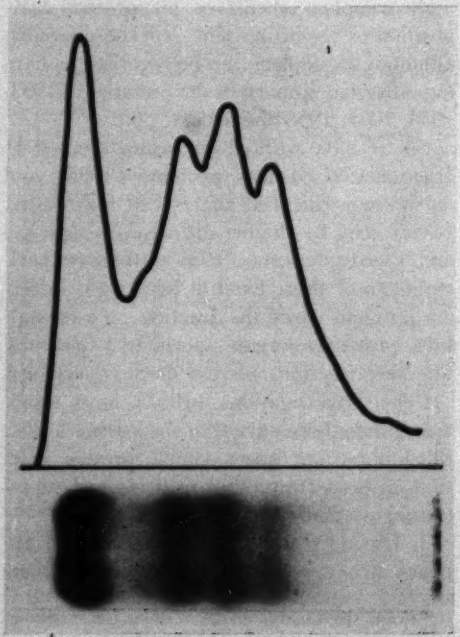

Fig. 1 (François and Rabaey). Paper electrophoresis. Child, aged three months. Total lens.

of varying age groups. The above-mentioned facts regarding the protein composition of the human lens pertain only to lenses from young children.

The results obtained by paper electrophoresis of lenticular extracts from adults are extremely disappointing (figs. 2 and 3). Even if, taking special precautions, one succeeds in carrying out an adequate electrophoresis on filter paper of an adult human lenticular extract, no separation is obtained, so to speak, but rather a more or less wide zone where the soluble protein has migrated.

Diagrams made on the basis of these electrophoreses sometimes seem to indicate various peaks; it is completely impossible, however, to separate these, let alone to attain a quantitative elaboration. It is even impossible to determine which of these zones correspond with the protein components found in young children. The various protein fractions in fact seem to migrate much closer together in adults than they do in children. Wherever fraction I (α-crystallin) can be characterized with some certainty the protein components best represented are seen to migrate very near this fraction, and in places where very little protein is found in the young child.

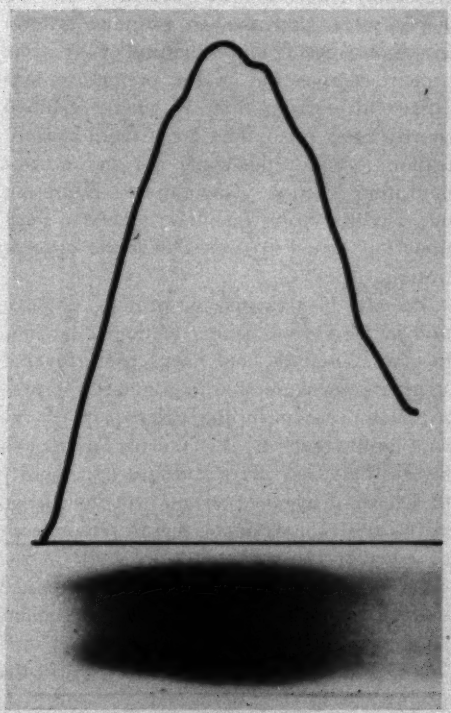

Fig. 2 (François and Rabaey). Paper electrophoresis. Adult, aged 42 years. Total lens.

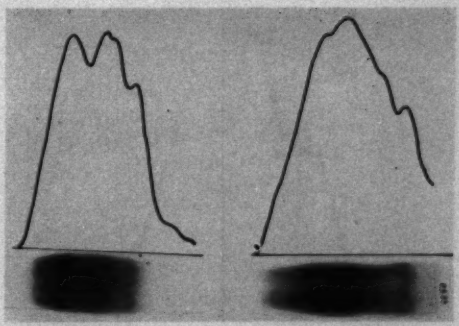

Fig. 3 (François and Rabaey). Paper electrophoresis. Adult, aged 42 years. (Left) Cortical part of the lens. (Right) Nuclear part of the lens.

This suggests that the adult lens contains chiefly proteins which differ from those contained in the young lens or that the proteins in the young lens are transformed in such a manner as to result in marked modifications in their mobility in an electric field.

All this clearly shows that the protein composition of the human ocular lens is subject to considerable changes during the individual's senescence. These changes appear to be very complicated, and the data obtained suggest, moreover, that protein patterns in adult human lenses show considerably more marked individual differences than those in children and adolescents.

An attempt was made to improve our understanding of these phenomena by investigating a series of lenses from subjects of various age groups.

Using the familiar pattern encountered in children as a starting-point, we hoped thus to be able to follow these changes step by step and to succeed in an adequate interpretation of the adult lenticular protein pat-

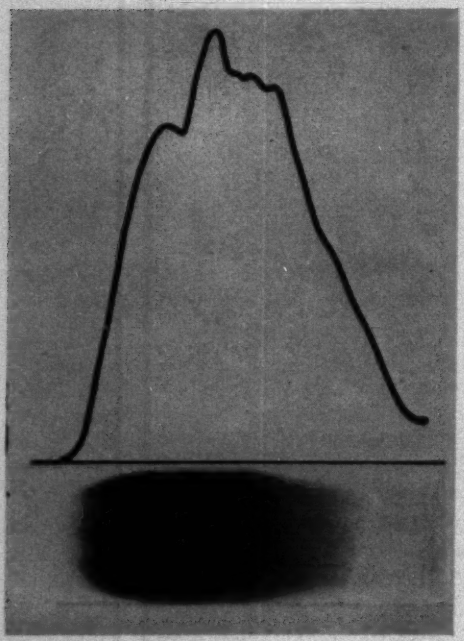

Fig. 4 (François and Rabaey). Paper electrophoresis. Adult, aged 24 years. Total lens.

tern. It should first of all be pointed out that we failed to find a complete solution of this problem with the aid of paper electrophoresis. In the discussion of results, therefore, results obtained by other methods have to some extent been taken into account.

An investigation of lenses from subjects of various age groups shows that the protein composition of the ocular lens is subject to a number of changes from an early age on. An investigation of subjects over 10 years of age is characterized by the fact that, at that age, orientation already is almost completely lost. It is a remarkable fact that the human ocular lens is so rapidly affected by what may be described as "senile changes." In this respect man differs considerably from many other mammals, for example, the horse which, at the age of 15 years, shows hardly any difference from the ocular findings in the young animal.

In the course of the first few years of life, the relative quantity of the slowest migrating fraction decreases; the concentration of fraction I (α-crystallin) meanwhile shows little variation. Apart from individual differences, it is obvious that the concentration of fraction II increases with increasing age.

After 10 years, quantitative fractionation of total lenticular extract has become extremely arbitrary (fig. 4). Yet it can be clearly seen that the protein components which migrate at the level of fraction II and slightly more toward the anode gradually increase in concentration, together with what seems to correspond with fraction III, and together seem gradually to constitute the most important part of the total lenticular protein. At the same time it is still difficult to distinguish the α-crystallin because it is markedly absorbed into the filter paper and no longer migrates as a symmetric peak. Increasingly less is seen of fraction IV.

We were also able to examine several lenses obtained from prematurely born. The protein composition of the premature lens differs little from that of lenses obtained shortly after birth. The α-crystallin concentration is about the same but the slowest migrating fraction (fraction IV) is present in a slightly larger quantity; whereas, fraction II is found in somewhat lower concentration.

We also investigated the protein composition in the various layers of the ocular lens in man. Since the ocular lens has a layered structure we were able to compare the protein composition in the older part of the lens with that in the freshly developed layers. This also offers a unique opportunity to determine whether certain proteins change at the site (for example, due to senescence) or whether other proteins are built in the outer layers. The protein patterns obtained with extracts of various layers of the ocular lens, moreover, are less complicated and more suitable for interpretation of the phenomena already described.

In the majority of cases, distinction is made between cortical and nuclear parts of

the lens. Especially in the case of younger lenses this distinction is rather an arbitrary one, although it is completely justified by the results obtained. In several cases the lens was divided into various layers which were separately extracted and submitted to electrophoresis.

In the case of infants and prematurely born infants, there is relatively little difference between the cortical and the nuclear part of the lens (figs. 5 and 6). Reliable data could only be obtained by investigating a large series of cases and by calculating the average value of several electrophoreses. The least marked difference was found as to fraction I (α-crystallin). Yet it is generally less large, percentagewise, in the nuclear region. The most striking and most constant difference concerned the slowest migrating protein fraction (fraction IV). This invariably showed a higher percentage concentration in the nuclear than in the cortical part. As to fractions II and III the possible significance of differences is often difficult to establish. It seems, however, that fraction II is percentage-wise smaller in the nuclear part than in the cortical part.

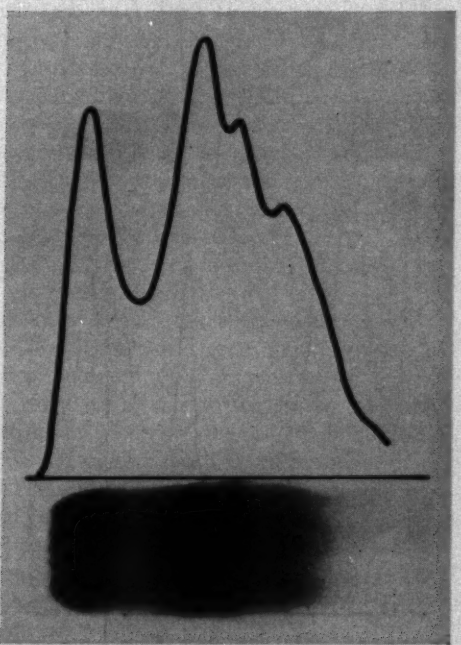

Fig. 5 (François and Rabaey). Paper electrophoresis. Child, aged 14 months. Total lens.

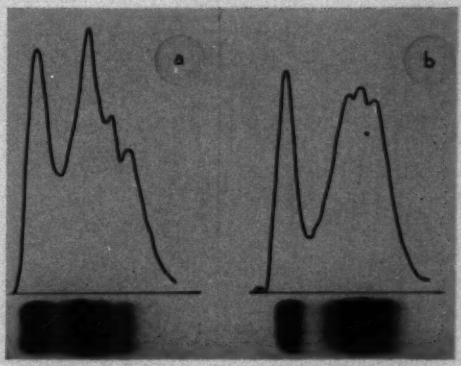


Fig. 6 (François and Rabaey). Paper electrophoresis. Child, aged 14 months. (a) Cortical part of lens. (b) Nuclear part of lens.

These conclusions are confirmed by investigation of lenses divided into three or four concentric layers. The results of paper electrophoresis of neonatal lenses showed that (figs. 7 and 8):

1. Fraction I (α-crystallin) is percentagewise slightly larger in the peripheral cortical part of the lens.

2. The percent concentration of fraction IV increases toward the nuclear part.

3. The ratio between fractions II and III is variable, fraction II prevailing in the cortical part, and fraction III in the nuclear part of the lens.

The percentages of the first three fractions are naturally influenced by the increase in fraction IV in the nuclear part. Separate calculation of the ratios of fractions I, II and III, and also of fractions II and III, thus putting aside the increase in fraction IV in the nuclear part, shows that:

1. The quantity of fraction I remains constant throughout the various layers of the young lens.

2. The ratios between fractions II and III vary in the cortical and the nuclear part of the lens. Fraction II predominates in the cortex, and fraction III in the nucleus.

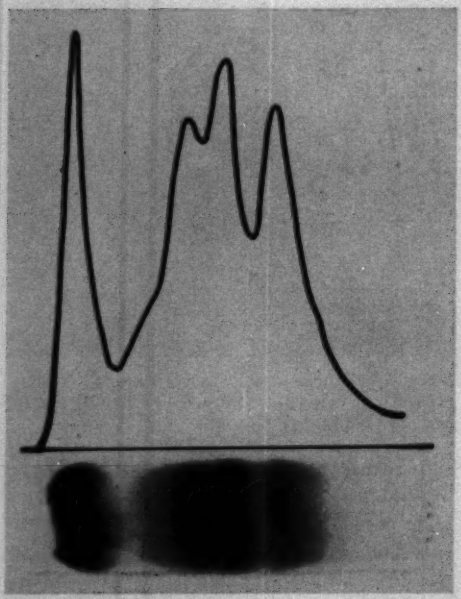

Fig. 7 (François and Rabaey). Paper electrophoresis. Newborn child, total lens.

Another important conclusion is that the nuclear part of the lens in infants shows about the same proportions as that of the premature lens.

Investigation, in a similar manner, of the lens of young adults (aged 24 years) (figs. 4 and 9), in which quantitative fractionation is still feasible to some extent, reveals as the most striking feature that the percentage of the slowest migrating fraction (fraction IV) is significantly lower than that in the neonatal lens. The fraction I shows about the same percentage, and fractions II and III a markedly higher percentage.

A study of the total quantity of the four soluble protein fractions (expressed in mg. N) present in the lens clearly shows that fraction IV has only slightly increased in the young adult as compared with the neonatal lens.

The neonatal lens contains virtually as much fraction IV as the adult lens. After birth its quantity shows but a very slight increase; whereas, the quantities of the other fractions increase after birth to about the same extent as does the total quantity of lenticular protein. Consequently, although the proportions in man are much more complicated, there exists in the human lens a protein, which is predominantly built up during the embryonic period. In man, too, it would be justified to use the term embryonic lenticular protein, which was indicated as the slowest migrating fraction (fraction IV) after paper electrophoresis.

An investigation of the percentage values of the protein fractions in the various layers

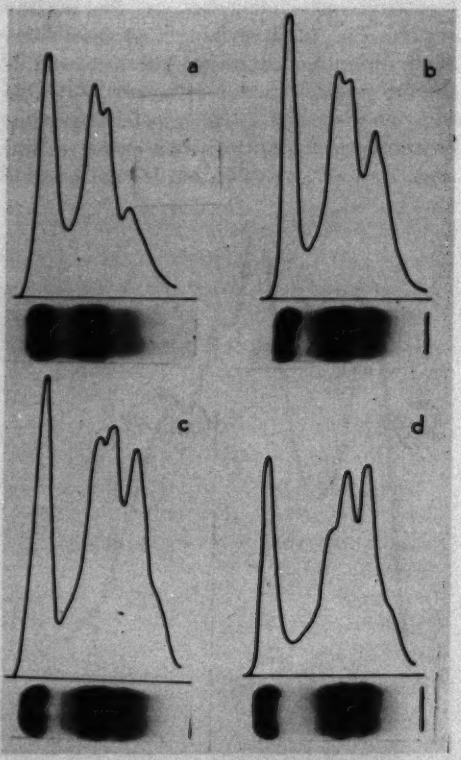


Fig. 8 (François and Rabaey). Paper electrophoresis. Newborn child. Lens divided in four concentric layers. (a) Outer cortical part. (b) Inner cortical part. (c) Outer nuclear part. (d) Inner nuclear part.

of the lens in young adults also shows that fraction IV is, for the most part, localized in the nuclear part of the lens; whereas its relative concentration in the cortical part is very low. Another striking feature is that fraction I (α-crystallin) shows a much higher percentage in the peripheral cortical part of the lens. The ratio of fractions II and III is variable, as in neonates. Fraction II predominates in the cortex, and fraction III in the nucleus.

So far it has been presupposed that the human lens contains only four different protein components, and that the fractions visible following paper electrophoresis constitute but a single protein. This hypothesis, although already more complicated than the previous conception of α-crystallin and β-crystallin, would be too simple.

By paper electrophoresis under certain conditions it was found that the three slowest migrating fractions were anything but homogeneous and at least showed asymmetries which were difficult to explain. A smaller, not sharply defined peak was more or less regularly found in front of fraction II (that is, toward the anode). Under certain conditions fraction II showed considerable broadening and seemed to include more than a single component. Under certain conditions, too, reduplication of fraction III offered difficulties, and fraction IV very often showed asymmetry towards the cathode.

A method was meanwhile developed* for ultramicro-electrophoresis in agar gel, which enabled us to investigate minute quantities of protein and to obtain electrophoretic patterns of embryonic lenses during the initial stages of evolution. It was soon found that this technique, apart from enabling us to demonstrate quantities of protein up to 0.10 μg., also insured much better fractionation of the various protein components of the ocular lens than filter paper electrophoresis. Paper electrophoresis has since been carried out purely as a routine.

* In collaboration with Dr. R. J. Wieme.

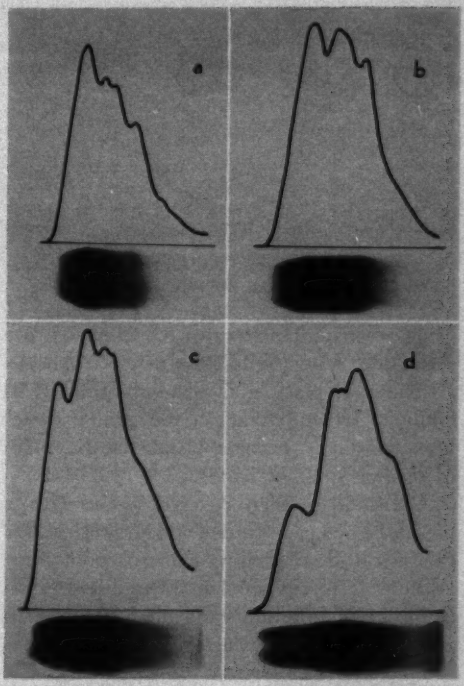


Fig. 9 (François and Rabaey). Paper electrophoresis. Adult, 24 years. Lens divided in four concentric layers. (a) Outer cortical part. (b) Inner cortical part. (c) Outer nuclear part. (d) Inner nuclear part.

### Technique of electrophoresis on agar

Liquefied, heated agar† is poured into a small glass disk or a microscope slide in such a way as to obtain a layer of 1.5 to 2.0 mm. The agar is allowed to solidify and to cool, after which the protein solution is introduced, with the aid of a capillary pipette, into a small excavation or, if the quantity of fluid is sufficient, into a small linear groove cut in the agar. This introduction is best done with the aid of a binocular microscope. The use of the linear groove is preferable because it ensures more exact quantitative estimation.

Electrophoresis requires 10 to 30 minutes,

† 1.25 percent dry agar (Bacto-Agar, Difco) in a buffer solution with veronal sodium, pH 8.4, ionic strength 0.05.

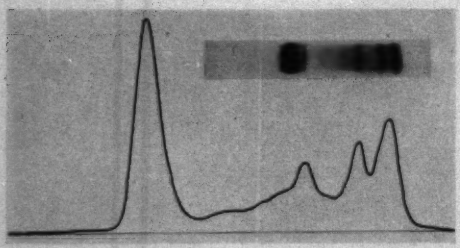

Fig. 10 (François and Rabaey). Agar electrophoresis. Child, aged 14 months. Total lens.

using a constant current of 8.0 to 12 ma. for each slide. After completion of electrophoresis the agar-plate is fixed* for at least 30 minutes. During fixation, the protein fractions gradually become visible in the form of opaque spots or bands.

The slide is then covered again with a piece of filter paper and dried at 37°C. After this, it can be treated as a microscopic preparation. It is stained for 30 minutes in an amido-black solution† and then washed in acid solution‡ until the agar background is completely decolorized throughout. The slide is then again dried, rendered transparent in toluol, and mounted in clarite.

The protein pattern is quantitatively read with the aid of a sensitive densimeter. The image of the slide, enlarged 20 times, is projected onto a depolarized glass bearing a slit diaphragm measuring 2.0 mm. and a very precise photo-electric cell (photomultiplier R.C.A. 931). The densities are plotted against the migration on millimetered paper. The density curves are then integrated with the aid of planimetry or by weighing the zones cut below the apex.

This method does in fact offer a number of advantages in lenticular protein determinations:

1. It is possible to arrange conditions so as to insure that only α-crystallin migrates to the anode relative to the line of application of the protein solution; whereas, the other fractions are drawn over a lesser or greater distance to the cathode by the flow of fluid (electro-endosmosic). This is very important in view of the fact that, in filter paper electrophoresis, the adsorption of α-crystallin in older lenses greatly impedes fractionation and impairs the accuracy of quantitative elaboration.

2. In agar gel the mobility of the various fractions (inasmuch as very large molecules are not involved, as in macroglobulinaemia¶) is surprisingly constant. It is possible to analyze several extracts under completely identical conditions on the same slide, for example, the cortical and the nuclear part of a lens of a young man and an old lens. This greatly facilitates comparison of the different protein components of the different extracts.

After agar gel electrophoresis of the lenticular proteins of an infant (fig. 10), at least 10 different protein components can be distinguished. The strongest fraction consists of α-crystallin, which migrates to the anode; even nearer the anode are a few very feeble fractions showing a very high rate of migration; these will be referred to as the *rapid fractions*. The concentration of these fractions increases with decreasing age of the lenticular fibers.

Toward the cathode there are two sharply circumscribed, highly concentrated fractions. Even nearer to the cathode a third component is seen, in very low concentration. These three fractions are referred to as the *slow fractions* I, II and III (III being the feeblest, nearest the cathode). The strongest (middle fraction) is referred to as *fraction E*.

The α-crystallin is separated from these *slow fractions* by five *middle fractions*, of which middle fraction IV is predominant in infants.

* Fixation fluid: 5.0-percent acetic acid in 70-percent ethanol.

† Staining materail:

| | |
|---|---|
| Amido-black 10 B | 0.5 gm. |
| Mercuric chloride | 5.0 gm. |
| Acetic acid | 5.0 ml. |
| Distilled water | 95.0 ml. |

(Filter before use)

‡ 5.0-percent acetic acid in distilled water.

¶ Dr. Wieme.

### Preliminary conclusions

We are still engaged, using this technique, in studying the changes in the protein composition of the ocular lens during senescence. General preliminary conclusions can be summarized as follows:

1. The older lenses contain no proteins other than those also found in infants, albeit in different proportions.

2. In the cortical part of the older lens the proteins show an anlage in different proportions to those in the younger lens. The nuclear part, however, shows changes which might be explained on the basis of conversion of the proteins themselves.

3. Within the first few years of life changes in α-crystallin are seen. The protein of the cortical part has a lower mobility than that of the nuclear part of the lens. In some cases there are even two separate peaks, one with higher and one with lower mobility.

Apart from the changes in α-crystallin already described, even infants show marked differences between the cortical and the nuclear part of the lens (fig. 11). The most striking feature is that, percentage-wise, the slow fraction II (fraction E) occurs in lower concentration in the cortical part; whereas, the middle fraction IV shows some predominance.

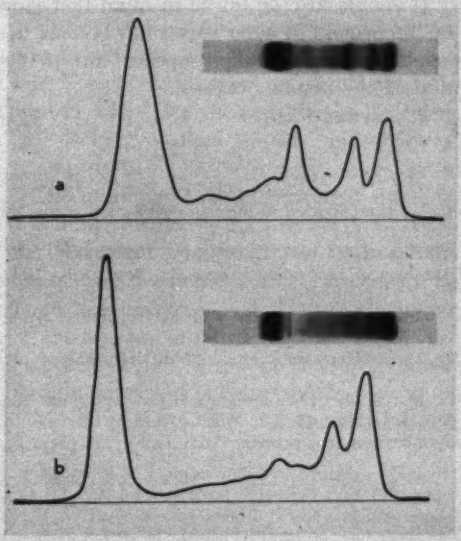


Fig. 11 (François and Rabaey). Agar electrophoresis. Child, aged 14 months. (a) Cortical part. (b) Nuclear part.

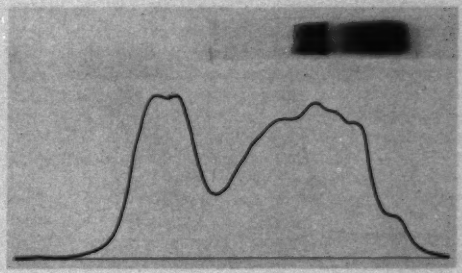

Fig. 12 (François and Rabaey). Agar electrophoresis. Adult, aged 25 years. Total lens.

During the first few years of life the relative concentration of fraction E shows some decrease. Middle fraction IV shows a relative decrease before the age of four years, whereas the other middle fractions are present in higher concentrations. At that time the differences between the cortical and the nuclear part of the lens have already considerably increased. Fraction E is relatively even lower in the cortical part as compared with the nuclear part. In the cortex some middle fractions come to the fore, while electrophoresis of the nuclear lens shows changes in the protein composition of the middle fractions.

In the adult lens (figs. 12 and 13) the electrophoretic picture is dominated almost entirely by the middle fractions, which constitute the majority of the lenticular proteins in this region. The relative concentrations of the slow fractions show a marked de-

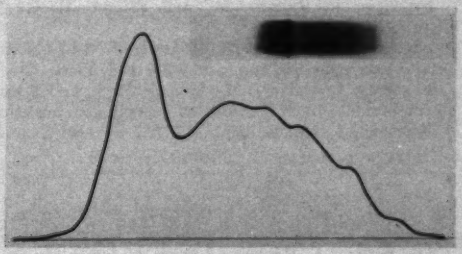

Fig. 13 (François and Rabaey). Agar electrophoresis. Adult, aged 42 years. Total lens.

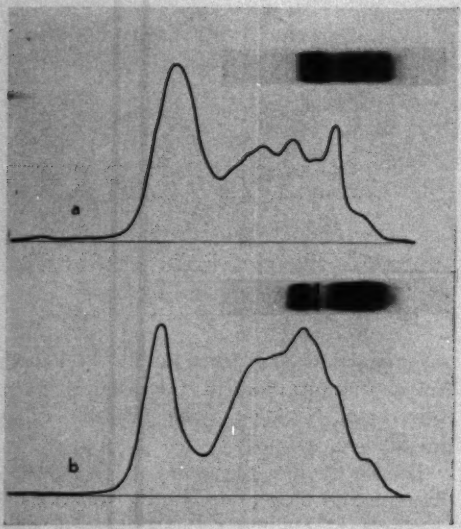


Fig. 14 (François and Rabaey). Agar electrophoresis. Adult, aged 25 years. (a) Cortical part (b) Nuclear part.

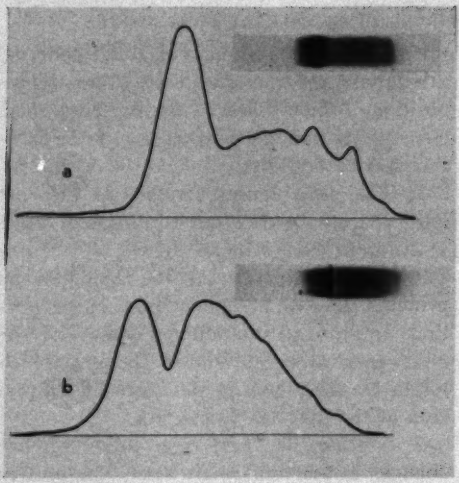


Fig. 15 (François and Rabaey). Agar electrophoresis. Adult, aged 42 years. (a) Cortical part. (b) Nuclear part.

crease, particularly that of fraction E. Subsequently slow fraction I also decreases. With senescence of the lens the middle fractions I, II and III come to the fore as compared with middle fraction IV, which was predominant in the infant.

In older lenses the cortical part is most similar to the aspects found in infants, with the exception of fraction E, which no longer constitutes anything remotely like an important percentage. The middle fractions, however, have also increased and become more important than those in infants. They are completely predominant in the nuclear part (figs. 14 and 15).

It should also be borne in mind that part of the protein in older lenses has become insoluble, which greatly impedes interpretation of the results obtained.

*2, Avenue Pasteur.*

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## Discussion

Dr. Robert A. Resnik: I think we have seen the results of some very fine work on the separation of lens proteins, tracing through the development of the lens as a tissue and as an organ. There are

some points that I would like to mention which are of general concern to me and which have general ramifications.

One of the points noted in this presentation is that there is present what might be referred to as an anlage or an "embryonic protein" in the lens. The implication that this protein might be the precursor of others or that there is interconversion of proteins in the lens is interesting but remains to be proven. From this standpoint the paper by Dr. François touched upon some rather fundamental points.

There is one thing that I would like to see done. Since most of this work is presented in the form of electrophoretic patterns on paper and agar gel, I feel that some reference material should be run concurrently so as to give some specific designation to these many fractions. This would enable investigators in other laboratories to know if they are dealing with the same fractions. Furthermore no attempt has been made to calculate mobilities. I am sure this could be done with the information available to the author in this case. This would certainly make it easier to compare results and be more definitive than we now are by calling something fraction 1 or fraction 2.

DR. ZACHARIAS DISCHE (New York): I would like to ask Dr. François about certain details of his work which were particularly interesting. I would like to ask whether he believes that some of these fractions that appear in smaller amounts might be due to associations between alpha and beta crystallin. Such association processes may influence very much the mobility of proteins, in the sense that an intermediate mobility might result.

I ask this question because certain observations on solubility of lens proteins suggest such associations. For instance, if lens proteins of cattle are precipitated with ethanol before any fractionation is carried out, then the total proteins later dissolve easily in water. This solubility disappears when a pure beta crystallin or if alpha crystallin fractions are tested in the same way. I think this would indicate the possibility that associations between the two proteins occur which could influence the solubility.

DR. JULES FRANÇOIS: I would like to say to Dr. Dische that in a study of immuno-analysis in double diffusion with serum antigens we have found that there are at least eight different proteins in the lens. That is the reason for thinking that the different fractions we found with this agar technique are very different fractions.

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# LENS PROTEINS*

## I. ALPHA CRYSTALLIN OF CALF LENS

ROBERT A. RESNIK, PH.D.
*Bethesda, Maryland*

The proteins of the lens have been of concern to many investigators. Amino acid analysis[1,2] and electrophoretic studies[3-6] have been reported for these proteins. However, little is known about the size and shape of these proteins.

The minimum molecular weight of alpha crystallin calculated by Krause[7] was based on incomplete amino-acid analysis. In the absence of detailed information concerning the measurements, it is difficult to know how to assess the value of 1,200,000 for the molecular weight of alpha crystallin based on light scattering measurements.†

As components of a transparent tissue it is possible that one or more of these proteins may be unique among proteins in some manner. Whether this is the case is not known, since very little quantitative information is available on this aspect. The purpose of this paper is to report the initial determination of various parameters which may be used to determine the size and shape of alpha crystallin obtained from calf lens.

### MATERIALS AND METHODS

All reagents used were of reagent grade or of the highest purity available. Ultracentrifuge runs were made at room temperature in a Spinco Model E ultracentrifuge at a speed of 59,780 revolutions per minute. Sedimentation coefficients were corrected to 20°C.

Diffusion measurements were carried out at 40°C. in a Spinco Model H electrophoresis-diffusion apparatus. A Tiselius type elec-

* From the Ophthalmology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service Department of Health, Education, and Welfare.

† These results were mentioned in the article by François et al.[8]

trophoresis-diffusion cell was used. The spreading of the boundary was followed by observing the Rayleigh interference fringes. In order to conserve material, after the boundary was sharpened initially siphoning was halted for about one to two hours. This was repeated three times until about 50 to 60 ml. of fluid had been withdrawn. Reference fringes were obtained by rotating the turret until the reference slits located at the center of the mask were in the optical path. The methods of both Longsworth[8] and Svensson[9] were initially used for treating the data. The same results were obtained using both methods of computation, but the method of Longsworth was preferred because it does not require that the center of the boundary be located. In addition, it is possible to determine the degree to which the boundary departs from an ideal Gaussian distribution.

The position of several pairs of fringes, $j_k$ and $j_l$ (columns 1 and 2 of Table 1), was measured using a comparator capable of reading to 0.01 mm. In the instrument used the magnification factor was equal to one. As a result the ratio of the vertical coordinates, H, of a pattern, to the height, h, in the channel, is 1. The computations, based on the assumption that the distribution in the boundary is ideal, are greatly simplified by using the "Tables of the Error Function and Its Derivative."[11] The value $(4Dt)^{\frac{1}{2}} \Delta H/\Delta h$, where t is the total elapsed time, is computed from the separation, $\Delta H$, (column 3, table 1) of the respective fringes. For a detailed account of this method of computation the article by Longsworth should be consulted. Zero time corrections were calculated by the method of Longsworth.[8] Diffusion coefficients were corrected to 20°C. by the relationship.[10]

$$\frac{D_1\eta_1}{T_1} = \frac{D_2\eta_2}{T_2}$$

where

$D_1$ = observed value of the diffusion coefficient
$\eta$ = viscosity of solvent
$T$ = absolute temperature

Density measurements were made in a pycnometer blown out of 7 mm. capillary glass tubing with a 2.0-mm. bore.

pH measurements were made on a Beckman Model G pH meter. Protein concentrations were based on the weight of material dried at 105°-110°.

## Preparation of alpha crystallin

In the initial attempts aimed at obtaining alpha crystallin the fractionation was followed by paper electrophoresis.

Preparations of alpha crystallin which

TABLE 1

THE TREATMENT OF FRINGE PATTERN DATA FOR 0.4-PERCENT ALPHA CRYSTALLIN AFTER DIFFUSION FOR 21,200 SECONDS

| $j_k$ | $j_l$ | $\Delta H = H_l - H_k$ | $\Delta h/(4Dt)^{1/2}$ | $(4Dt)^{1/2}\Delta H/\Delta h$ |
|---|---|---|---|---|
| 2 | 35 | 0.229 | 1.4051 | 0.1629 |
| 4 | 40 | 0.211 | 1.3159 | 0.1603 |
| 6 | 46 | 0.209 | 1.3162 | 0.1587 |
| 8 | 47 | 0.202 | 1.2640 | 0.1598 |
| 10 | 49 | 0.196 | 1.2353 | 0.1586 |
| 12 | 51 | 0.197 | 1.2248 | 0.1608 |
| 14 | 53 | 0.198 | 1.2306 | 0.1608 |
| 16 | 55 | 0.204 | 1.2537 | 0.1627 |
| 18 | 57 | 0.213 | 1.2983 | 0.1640 |
| 20 | 59 | 0.228 | 1.3750 | 0.1658 |
| 25 | 61 | 0.239 | 1.4255 | 0.1676 |
| 30 | 63 | 0.292 | 1.7235 | 0.1694 |
| | | | | 0.1625 av. |

$D_4^\circ = 1.079 \times 10^{-7}$ uncorrected
$D_4^\circ = 1.067 \times 10^{-7}$ corrected for zero time
$D_{20}^\circ = 1.78 \times 10^{-7}$

were thought to consist of one component alone on the basis of paper electrophoresis were not free of small amounts of a component which had a lower sedimentation coefficient. For this reason paper electrophoresis was found to be inadequate as the sole means for unequivocal estimation of the degree of homogeneity of a preparation. Although many kinds of paper were tried and the pH varied, in the hands of this investigator at least, paper electrophoresis seemed to be of limited value in this specific instance. As a result, the state of preparation of alpha crystallin was routinely followed by use of the analytical ultracentrifuge.

The method of preparation that has been used involves a treatment of the aqueous solution obtained from calf lens cortex. Decapsulated lenses were stripped of their cortex on a cold stage and homogenized in an equal volume of ice-cold distilled water using an iced glass homogenizer. After centrifuging the homogenate for 30 minutes at about 15,000 to 20,000 × G, the supernatant solution was adjusted to pH 5.0 by the slow dropwise addition of 0.1 *M* HCl with continuous stirring in a cold room. The pH was measured on aliquots, from time to time, at room temperature. The precipitate was removed by centrifugation. This precipitate is the alpha$_1$ fraction of François et al.[5] Absolute alcohol was added with continuous stirring to bring the concentration of the supernatant solution to 13 percent with respect to alcohol.

This precipitate, alpha$_2$ of François, was removed by centrifugation. The addition of alcohol at a temperature greater than 0°C. is important because at 0°C. other proteins are precipitated along with the alpha crystallin. Alcohol has been added both to solutions in a refrigerated bath at a temperature of 5°C. to 10°C. as recommended by François[5] and to solutions in a cold room whose temperature is set for 3°C. to 4°C. In both instances the same degree of separation was obtained.

Both the materials precipitated at pH 5.0 and in 13-percent alcohol were suspended in water and dissolved by slow addition of 0.1 *M* NaOH with continuous stirring for a few hours. The pH of the solution was between pH 6.5 and 7.0. The two fractions were reprecipitated two or three times.

The supernatant solution, after precipitation at pH 5.0, was added to the solution of the material insoluble at an alcohol concentration of 13 percent. In each instance, adjusting the solution of the alcohol insoluble material to pH 5.0 resulted in the formation of a precipitate. On the basis of solubility at pH 6.5 to 7.0 it does not seem apparent that any material has been denatured.

At this point in the procedure there still remained a small amount of a slowly sedimenting component. The solutions were then dialyzed against many volumes of distilled water for 24 to 48 hours during which time a precipitate appeared. The solutions of water soluble material were then lyophilized. The resulting preparation appears as a single sedimenting component with a sedimentation coefficient of 18.8 × $10^{-13}$ (fig. 1).

Fig. 1 (Resnik). Sedimentation pattern of 0.4-percent alpha crystallin in 0.5 *M* NaCl. Bar angle setting —45°C. Picture taken after 12 minutes at 59,780 rpm.

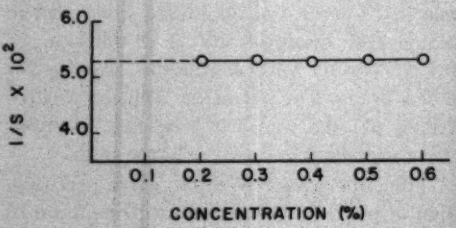


Fig. 2 (Resnik). Plot of the reciprocal of the sedimentation coefficient versus concentration for alpha crystallin. In pH 7.5 sodium barbital buffer, μ-0.05, sodium chloride, μ-0.20.

## Results and Discussion

Since material could be precipitated from a solution at pH 5.0 by the addition of alcohol it would appear that the addition of alcohol is necessary only to obtain complete precipitation of the alpha crystallin from solution. In view of this the notation of François for $alpha_1$ and $alpha_2$ is misleading. Furthermore, in a single preparation both "$alpha_1$" and "$alpha_2$" fractions had identical sedimentation coefficients (table 2).

### Sedimentation

In Table 2, it can be seen that the protein sediments at the same rate in both buffered and unbuffered salt solutions.

The sedimentation rate of proteins is sensitive to concentration effects. With decreasing protein concentration the rate generally increases. This type of relationship was observed in several experiments. However, in other instances there was no apparent concentration dependence (fig. 2). The reason for these different results is not apparent and must await further investigation of this phenomenon.

TABLE 2

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|---|---|---|---|
| 1 | Extrapolated to zero concentration | 18.8 | μ−0.05 Barbital-Acetate buffer ρH 7.5 μ−0.20 NaCl |
| 2("α2") | 0.5 | 18.8 | 0.5 *M* NaCl |
| 2("α1") | 0.27 | 19.3 | 0.5 *M* NaCl |
| 3("α1") | 0.5 | 16.5 | 0.5 *M* NaCl |
| 3("α2") | 0.5 | 16.4 | 0.5 *M* NaCl |

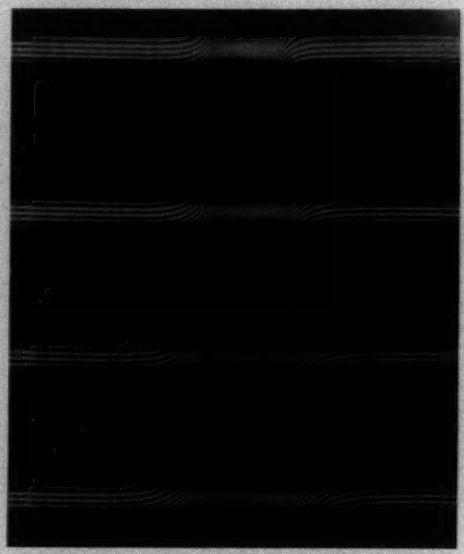

Fig. 3 (Resnik). Diffusion of 0.3-percent alpha crystallin in pH 7.5 sodium barbital-acetate buffer, μ-0.02 and NaCl, μ-0.2. The patterns, reading from top to bottom, were those obtained after 24, 46, 70, and 94 hours of diffusion. Solution is on the left side, solute on the right side of the boundary as pictured here.

### Diffusion

As mentioned earlier, the method used for computing the diffusion coefficients provides an index of the extent to which the boundary deviates from a normal distribution. In the event that the distribution is ideal, the figures in Column 5 of Table 1 should be constant. It is readily seen that there is some deviation from ideality at one side of the curve. This deviation appears as a more gradual slope in the fringes conjugate to the solution in Figure 3.

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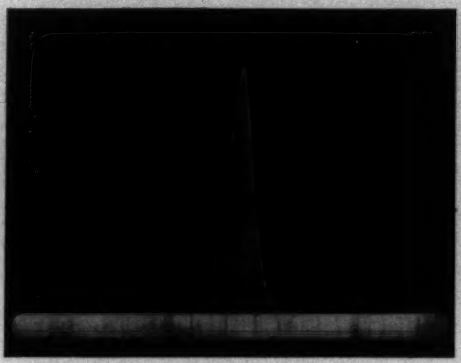

Fig. 4 (Resnik). Moving boundary electrophoresis Schlieren pattern of 0.8-percent alpha crystallin in pH 8.6, μ-0.1, sodium barbital-acetate buffer. Ascending limb. Picture taken after three hours.

## Molecular weight of alpha crystallin

Substituting into the Svedberg formula,[12] values of $18.8 \times 10^{-13}$ for $S_{20}$, $1.78 \times 10^{-7}$ for $D_{20}$ and 0.74 for $V_{20}$, a molecular weight of 1,000,000 is obtained. This is slightly lower than that recently reported.[5] This might be due to the fact that the value one obtains from light scattering gives the weight average molecular weight which can be greatly influenced by the presence of even a small amount of a species of higher molecular weight. In fact on the basis of the skewness of the sedimentation pattern in Figure 1 it would be anticipated that the molecular weight as calculated from light scattering data should be greater than that calculated from sedimentation and diffusion data. Using the same preparation, for which a molecular weight of $1 \times 10^6$ was calculated on the basis of sedimentation and diffusion data, a molecular weight of about $1.5 \times 10^6$ was estimated by light scattering.

## Shape of alpha crystallin

The ratio of the frictional coefficient, f, for alpha crystallin, to the frictional coefficient for an unhydrated sphere of the same size, $f_0$, is 1.78. This frictional ratio is higher than anticipated on the basis of a lack of concentration dependence during sedimentation. Assuming that hydration alone would account for an $f/f_0$ value of 1.12,[13] it appears that alpha crystallin is an asymmetric molecule.

At this point the only conclusion that may be reached is that alpha crystallin has a molecular weight of the order of magnitude of one million.

*Ophthalmology Branch (14).*

## References


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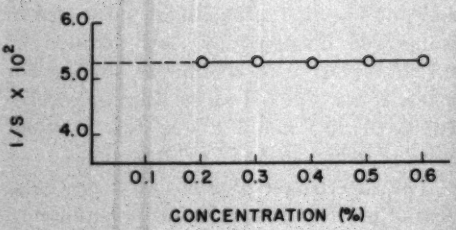


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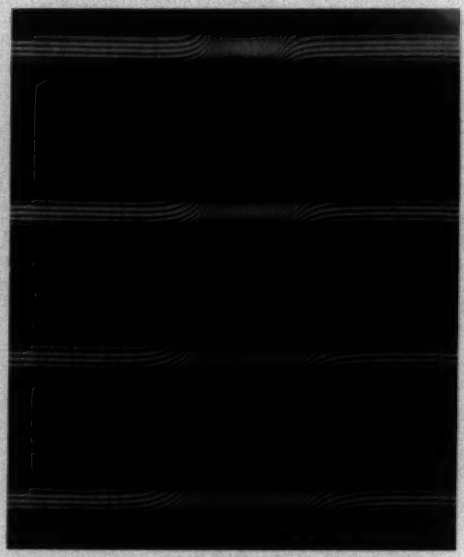

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## Discussion

Dr. Endre A. Balazs (Boston): I want to compliment Dr. Resnik on the fine paper which he has presented. This is probably one of the first of what I hope will be a series of papers on the physicochemical characterization of lens proteins. In view of Dr. François' report, one can expect that the characterization of lens proteins may take some time, since their number seems to increase as more thorough investigations are made on the lens.

I really don't have too much to say about Dr. Resnik's paper. He has already covered all possible speculations that could be made from his data. As he pointed out, it is quite surprising and very interesting to note that the sedimentation constant of his lens protein does not depend upon the concentration. This is especially interesting because the molecular weight calculated from the diffusion and sedimentation data is quite high. A more complete analysis of the diffusion constant might better explain this observation.

Light-scattering studies on this protein would be of great value. From the light-scattering data one could calculate the probable shape of the molecule, which might possibly explain the very low concentration dependence of the sedimentation constant.

Finally, I should like to mention that at this stage in the investigations it is probably more important to collect physicochemical data on the lens proteins than to try to compute the definite size and weight of the molecule based on assumptions regarding hydration, shape and molecular interactions. In the final analysis, the hydration, shape and interaction of the molecules will determine the parameters derived from sedimentation, diffusion and other physicochemical measurements. In order to eliminate the errors caused by molecular interaction the physicochemical constants should be measured at various concentrations and extrapolated to zero concentration. The dependence of these various physicochemical constants on concentration and on such factors as the type of ionic milieu and ionic strength often yields more information about the characteristics of the molecules than the calculated size alone.

Again, I should like to compliment Dr. Resnik on his fine work and express the hope that he will continue these investigations.

Dr. Robert A. Resnik (closing): I want to thank Dr. Balazs for his comments, and I just wish to mention that as yet there is no evidence that the proteins of the lens are unique, yet one would expect, being components of a transparent medium, that they might have some uniqueness and some particular property. To this we will continue our endeavors.

# STUDIES OF BRUCH'S MEMBRANE*

## Flow and permeability studies in a Bruch's membrane-choroid preparation

Wood Lyda, M.D., Niles Eriksen, Ph.D., and Narendra Krishna, M.B.
*Seattle, Washington*

Bruch's membrane assumes importance in the physiology of the eye because of its strategic position. On its outer surface is the highly vascular choriocapillaris. On its inner side are the pigment epithelium and the rods and cones of the retina. Most of the nutrition of the pigment epithelium, the layer of rods and cones and the outer nuclear layer of the retina is derived from the choriocapillaris and must pass through this laminated, collagenlike membrane to reach these inner structures.

This initial report represents the results of some of our studies with the membrane and details the technique we used in obtaining these results. It is hoped that it will stimulate the interest of others in this important, but long neglected, membrane.

### Bruch's membrane preparation

Dog eyes were used in the experiments because they were readily available, had a definite Bruch's membrane and were adapt-

* From the University of Washington Medical School. This study was supported by funds from grant B-845 of the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland, and by the Department of Pathology, University of Washington Medical School.

able to the dissecting technique used in isolating the membrane.

The membranes were prepared from freshly enucleated eyes by making two scleral incisions. One was made around the ora serrata and the second was extended meridionally to the optic nerve. The sclera was dissected from the underlying choroid along the plane of the suprachoroidal space with great care, so as not to tear the choroid. The exposed choroid was incised just posterior to the ora serrata and carefully detached from the retina.

The choroid, with Bruch's membrane, was placed in a flat dish containing sterile water and studied under the Disectoscope. The pigment epithelium overlying Bruch's membrane was removed under magnification by macerating and gently rubbing the surface of the membrane with a glass rod. The choroid attached to the membrane was removed by cutting away the larger tags with scissors and then macerating and rubbing with a glass rod.

This dissecting technique removed all of the pigment epithelium and approximately two-thirds of the choroid. The inner one third of the choroid could not be removed without tearing Bruch's membrane because of the interdigitation of the pigment cells of the choroid with extensions of the elastic lamina of the membrane.

The choroid that remained attached to Bruch's membrane probably had some influence on the flow rates through the preparation. However, the amount of choroid that did remain attached was a constant factor in all preparations and did not seem to influence comparative results.

In the early stages of the experiment fresh membranes were used for only a few hours. We soon found that with the experimental procedures that were employed membranes as old as six days responded in essentially the same manner as fresh preparations.

Histologic sections were made of approximately 50 of the over 200 membranes that were prepared (fig. 1). In all of the sections Bruch's membrane and the attached choroid were edematous. There were no breaks found in Bruch's membrane in any of the sections.

Membranes from the eyes of two albino dogs were found to give the same results as the pigmented preparations.

## Apparatus

In order to study the flow rates of aqueous solutions through the membrane a device considered to be essentially leak-proof was designed to hold the tissues. The device was constructed of lucite and consisted of two threaded portions that when screwed together formed a cylindrical tube seven mm.

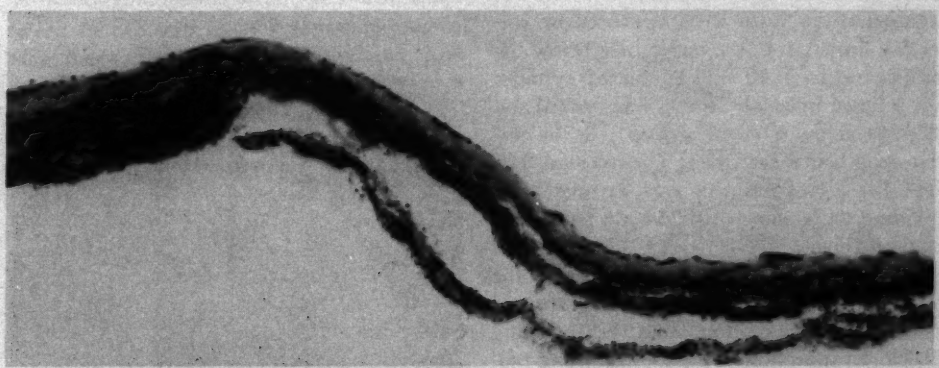

Fig 1 (Lyda, Eriksen, and Krishna). A histologic section of a posttest membrane, showing edema and thickening of Bruch's membrane and the small portion of attached choroid.

Fig. 2 (Lyda, Eriksen, and Krishna). The device designed to hold the membrane consists of two threaded lucite portions, a perforated disc and a washer.

in diameter (fig. 2). The membrane was placed on a centrally perforated disc and a second disc with a seven-mm. opening, to coincide with the perforations of the first, was placed on top. The flat inside surfaces and threads of the holder were lightly coated with vaseline. The discs, with Bruch's membrane facing upward through the seven-mm. opening, were placed in the lower part of the holder and the threaded pieces tightened.

The holding device was attached by means of a plastic sleeve to a 250 ml. reservoir with a short outlet tube of the same diameter as the tube leading to the membrane. Four of these membrane preparations were used simultaneously (fig. 3).

### Experimental procedure

In carrying out flow rate studies the reservoir and the tubes above and below the membrane were rinsed and filled with a test solution. Flow rates were measured by collecting drops from the outlet tube below the membrane in a 10 ml. graduated cylinder for a timed interval. Timing was started and stopped at the moment a drop fell, the initial drop being discarded. The average flow rate for an experiment was calculated by dividing the volume of fluid collected by the elapsed time.

The recorded flow rates represent the difference between the actual flow rate and the rate of evaporation from the collecting drops. Evaporation from the collecting cylinder and from the reservoir appeared to be relatively insignificant. However, evaporation from the collecting drops had the effect of accentuating the difference between fast and slow rates, but not to the extent of invalidating the qualitative interpretation of the results.

The flow rate characteristics of each membrane preparation were assessed with distilled water before further tests were performed. A flow rate greater than 7.0 microliters per minute was arbitrarily considered indicative of a leaky membrane and the membrane was discarded.

The flow rates through the membranes were sufficiently slow to permit the maintenance of a constant pressure head above the membrane with a minimum of adjustment during a test period. In all of the experiments the pressure head of the test solution was maintained at 12 cm. of water. Some of our observations indicated that increases and decreases in the pressure head caused corresponding changes in the flow rate.

Pilot studies of the effect of relatively large temperature variations indicated decreases in flow rates with decreases in temperature. These studies were abandoned with the realization that the temperature of the test liquid could not be varied without concomitant changes in its other physical properties, notably viscosity. For this reason all experiments were conducted at room temperature, with a maximal daily variation of approximately 2°C.

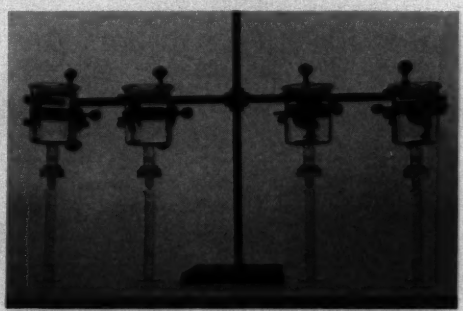

Fig. 3 (Lyda, Eriksen and Krishna). Four apparatuses were used in the experiments.

In a typical flow rate experiment four membrane preparations were tested with distilled water for two periods of 24 hours. Three of the membranes were then tested with different concentrations of a biologically occurring substance while the fourth was retained as a control. After three flow periods of 24 hours, the test solutions were replaced by water and additional 24-hour runs were made. Whenever the solutions were changed the water in the control preparation was also changed because the manipulation of solutions was usually followed by a temporary increase in flow rate. Except for this temporary phenomena the control flow rates tended to increase gradually during the course of an experiment. Since no two membranes behaved identically under seemingly identical conditions the controls served only as a general aid in the interpretation of the results.

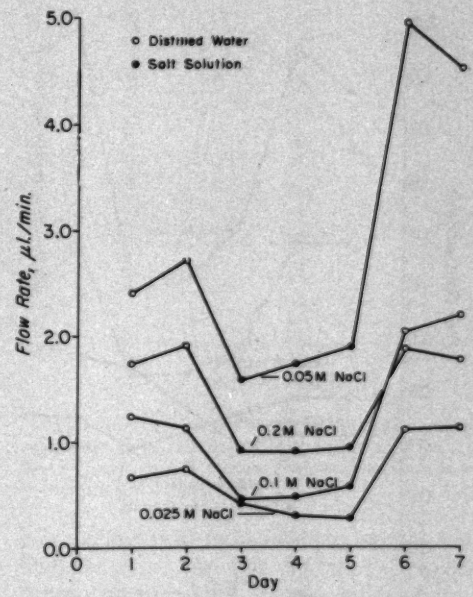


Fig. 4 (Lyda, Eriksen and Krishna). The flow rates of solutions of 0.025, 0.05, 0.10 and 0.20 molar NaCl all decreased and then rose with water.

## Results

### Effect of sodium and potassium chloride

NaCl in concentrations of 0.025, 0.05, 0.10 and 0.20 molar at pH 5.7 ± 0.2 produced a definite decrease in flow rates as compared to water (fig. 4). When the salt solutions were replaced by water the flow rates rapidly returned to their initial value or exceeded them. The results with KCl were similar. The differences in effect between the two salts appeared to be no greater than the variations observed with the control preparations.

When the concentrations of the NaCl or KCl solution passing through the same membrane were increased stepwise there was also a decrease in flow rates. This decrease was definite at 0.20 molar and maximal between 0.05 and 0.10 molar. Solutions of greater concentration did not decrease the flow rates further.

### Effect of glycine

Solutions of glycine in concentrations of 0.05, 0.10 and 0.20 molar (pH approximately 6.0) when perfused through the membrane resulted in decreased flow rates (fig. 5). This effect began with the first run of the 0.20 molar solution but was delayed until

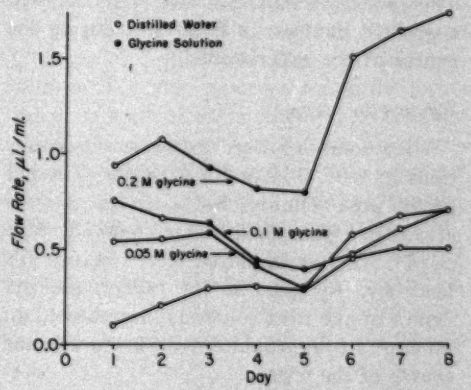


Fig. 5 (Lyda, Eriksen and Krishna). The flow rates of solutions of 0.05, 0.10 and 0.20 molar glycine decreased. Distilled water was used as the control solution and caused an increase in flow rate in a posttest run.

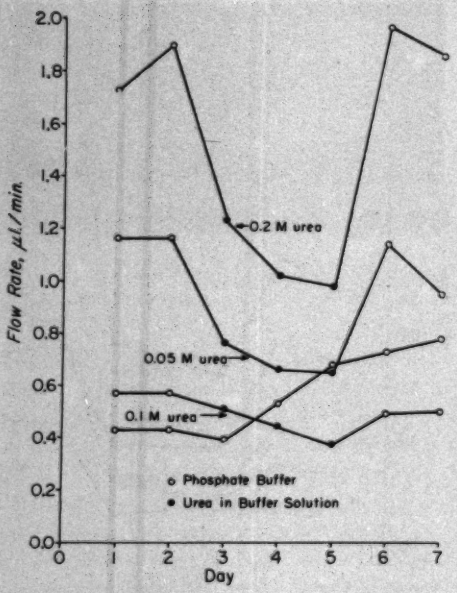


Fig. 6 (Lyda, Eriksen and Krishna). The flow rates of solutions of 0.05. 0.10 and 0.20 urea decreased. Phosphate buffer was used as the control and diluting solution.

the second run with the lower concentrations. All of the flow rates eventually returned to or exceeded the initial values when the glycine solutions were replaced by water. The control membranes with water showed a gradual increase in flow rates during the course of the experiments.

## Effect of urea

Urea solutions were tested in concentrations of 0.05, 0.10 and 0.20 molar. The pH of the urea solutions had a tendency to increase so a phosphate buffer (0.002 M Na) at pH 5.5, was employed to counteract this tendency. In spite of the buffer, the pH values of the urea solutions increased to an average of approximately 6.2 during the course of the tests.

Following the initial runs through the membranes with phosphate buffer, at pH 5.8, all of the urea solutions produced a decrease in flow rates (fig. 6). When the urea solutions, at pH 6.2, were replaced with phosphate buffer at pH 6.2, the flow rates increased. The curves for 0.05 and 0.20 molar urea followed essentially parallel courses in that both showed a fairly sharp decline in flow rate with the introduction of urea and a sharp increase after its removal, with a second decrease during the second post-test run. The curve for the 0.10 molar urea, for unknown reasons, was much flatter than the others.

## Effect of glucose

The perfusion of glucose solutions through the membranes in concentrations of 0.05, 0.10 and 0.20 at a pH of approximately 6.0 resulted in decided decreases in flow rates (fig. 7). When the glucose solutions were replaced by water the flow rates remained depressed instead of regaining their initial value.

During the course of the test the pH of the 0.10 molar solution decreased to pH 5.5 and that of the 0.20 molar solution to pH 5.0. These changes might have been greater at a constant pH, as indicated by studies on the effect of pH variations.

## Effect of pH variation

Pilot studies of solutions at various pH values indicated that flow rates through the

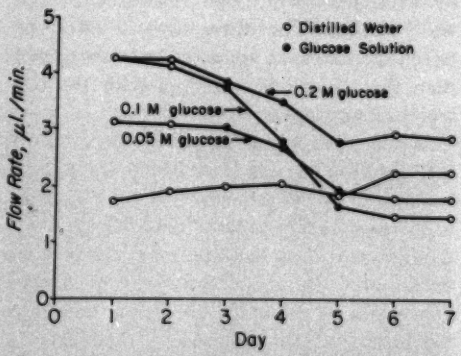


Fig. 7 (Lyda, Eriksen and Krishna). The flow rates of solutions of 0.05, 0.10 and 0.20 molar glucose were decreased. There was no increase when water was used in posttest runs.

membrane were depressed at both high and low pH values. A series of experiments were carried out with solutions in the pH range of 3.5 to 8.9 in order to find the pH of maximal flow rate. Reference flow rates of solutions of 0.10 molar NaCl at pH 5.9 were obtained for two membrane preparations. One series of preparations were serially tested at increasing pH values and the other at decreasing pH values. Alkaline pH values were obtained by the addition of small amounts of sodium acetate, sodium bicarbonate and sodium carbonate. Acid pH values were obtained by the addition of traces of HCl. The ionic strength of the solutions was maintained at 0.1. Two 24-hour runs were made for each pH value and the flow rates corresponding to the second run for each pH was used to construct a graph correlating flow rates with pH (fig. 8).

The flow rate was found to be maximal between pH 5.3 and pH 4.7. Below pH 4.7 there was a precipitous drop in flow rate while above pH 5.3 there was a definite, but less marked, decrease.

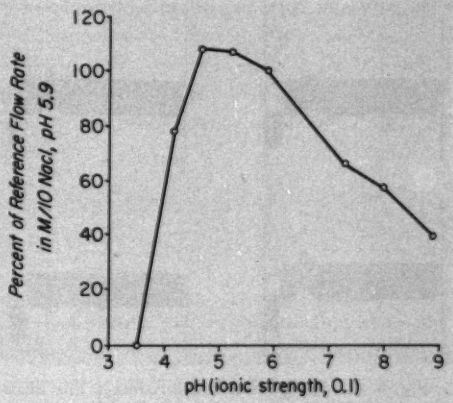


Fig. 8 (Lyda, Eriksen and Krishna). The graph represents flow rates of a 0.10 molar solution of NaCl at various pH values. The maximal flow rate is between pH 4.6 and pH 5.3.

### Molecular permeability

In order to estimate the upper size limit of particles that would pass through the membrane four human proteins, urinary myeloma protein, oxyhemoglobin, serum albumin and serum myeloma gamma globulin, were tested. The molecular weights of the hemoglobin and the serum albumin were assumed to be in the range of 65,000 to 70,000. The molecular weight of the urinary protein and the serum gamma globulin were estimated by ultracentrifugal sedimentation analyses to be approximately 45,000 and 150,000, respectively. The myeloma gamma globulin solution was a dilution of unfractionated serum in which 65 percent of the protein, as determined by a moving boundary electrophoretic analysis at pH 8.6, was a homogeneous component in the gamma globulin range. The other protein preparations were essentially pure. Stock preparations of the four proteins were equilibrated by dialysis against 0.10 molar sodium acetate and diluted before use with the same strength acetate solution. All of the solutions remained at a pH of 7.1 $\pm$ 0.1 during the experiment. The procedure that was followed was the same as that used in the other flow rate studies except that the outlet tube below the membrane was filled with pure solvent at the start of each experiment.

Analysis of the solutions in the outlet tube below the membrane, after two or three days of flow, showed that the urinary protein (0.25 gm./100 ml.), hemoglobin (0.10 and 0.05 gm./100 ml.) and albumin (0.05 gm./100 ml.) readily passed through the membrane in spite of the markedly reduced flow rates. The hemoglobin solutions were analyzed colorimetrically and the other protein solutions were analyzed ultracentrifugally. The passage of the myeloma gamma globulin (1:100 dilution of the original serum) was definitely retarded. The concentration of gamma globulin below the membrane barely exceeded the limit of detectability (fig. 9).

### Discussion

Under our experimental conditions all of the dilute aqueous solutions tested decreased

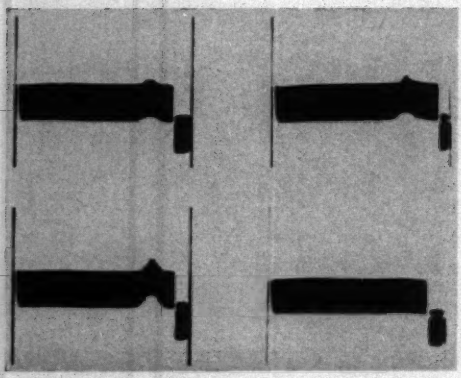

Fig. 9 (Lyda, Eriksen and Krishna). The ultracentrifuge tracings on the left are of serum albumin and those on the right for serum myeloma globulin. The upper tracings are from solutions above the membrane and the lower from those below the membrane.

flow rates through the membrane. Factors possibly affecting the flow rates are: viscosity, particle size, swelling of the membrane and interaction of the solutes with the membrane.

It does not seem likely that the viscosity increases of the test solutions, in the concentrations used, were sufficient to have more than a slight effect. Moreover, dilute solutions of KCl are slightly less viscous than water at 25°C. but the decrease in flow rates they produced were of the same order as that found with other test solutions.

In view of the ease with which hemoglobin and albumin flowed through the membrane it seems unlikely that the particle size of salts, urea, glycine and glucose in relation to pore size, would play a significant role in decreasing flow rates.

It would appear from our studies that there is an unknown interaction of solutes with the membrane. This is suggested by the difference observed between the effect of solutions of salt and glucose on flow rates. Both NaCl and KCl produced sharp decreases in flow rates followed by a rapid increase when the solutions were replaced by water. The effect of the glucose was not as immediate in reducing flow rates as were the salts and there was almost no increase in flow rate when glucose was replaced with water. A possible explanation of this phenomena is that glucose is slowly absorbed by the membrane and is gradually dissipated by water. An alternative, but less likely, explanation is that glucose produces an alteration in the chemical structure of the membrane.

The zone of maximal flow rate between pH 4.7 and pH 5.3 corresponds to an isoelectric point for the membrane in the vicinity of pH 5.0. This value compares favorably with the isoelectric point of other collagens as reported by Gustavson.[1]

It is interesting to note that Friedenwald and Stiehler[2] found an isoelectric point of pH 5.48 for the epithelial-stromal barrier of the ciliary body. They also stated that their observations indicated that the ciliary body barrier is a material substance and not merely a bounding surface, although there is no histologic evidence of a continuation of Bruch's membrane to the area.

An isoelectric point between pH 4.7 and pH 5.3 for Bruch's membrane-choroid preparation would indicate that a physiologic pH of 7.2 the membrane would be negatively charged. Under these circumstances we could speculate that Bruch's membrane may act in much the same way as the stromal-epithelial barrier of the ciliary body.

Our experiments indicate that molecules of the size and shape of human hemoglobin and serum albumin, with a molecular weight between 65,000 and 70,000, readily pass through the membrane. Molecules of the gamma globulin, having a molecular weight of approximately 150,000, were retarded in their passage through the membrane. Although 150,000 seems to be the upper molecular weight limit of molecules that permeated the membrane under our experimental conditions, other substances with similar or greater molecular weights but with different chemical constitution and molecular configuration might be able to pass through.

## Summary

The results of perfusion and permeability studies through a Bruch's membrane-choroid preparation indicates that there are important physiologic properties of the membrane that are worthy of further study. The effect of solutions of salt, urea, glycine and glucose on the membrane were studied. The study of the effect of solutions with variable pH on the membrane were also undertaken.

Permeability experiments indicated that the limit of molecular size that would pass through the membrane was in the range of 150,000.


*706 36th Avenue (22).*

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1. Gustavson, K. H.: The Chemistry and Reactivity of Collagen. New York, Academic Press, 1956.
2. Friedenwald, J. S., and Stiehler, R. D.: Circulation of the aqueous: VII. A mechanism of the secretion of the intraocular fluid. Arch. Ophth., **20**:761 (Nov.) 1938.


## Discussion

DR. PETER C. KRONFELD (Chicago): As far as I know, this is the first experimental study of the lamina vitrea as a biologic membrane. Since it is interposed between visual epithelium and its very highly specialized blood supply, the functional properties of this membrane are of considerable importance.

The essayists, I believe, have demonstrated that Bruch's membrane can be isolated quite satisfactorily with a minimum of trauma to the membrane itself. The extraneous choroidal tissue that stays with the membrane is in all probability, made up of the same chemical materials as the membrane itself, since the extraneous tissue represents the extensions of the lamina lastica into the stroma of the choroid. Therefore, I would not be worried too much about the little bit of admixture of choroidal elements.

As a first step in the investigation of the function of this membrane, the essayists have used the method of filtration rates, which is a well-established method of testing membranes. It is not necessarily the most physiological method.

One might conceive of obtaining more clear-cut information by placing this membrane in the little chamber that Dr. Ussing designed for his work on the isolated frog skin. We have all been very much impressed with the results obtained by that method.

Transfer rates of ions and colloids, particularly with the help of tracer substances, can be determined very accurately with that setup. It is my feeling that the membrane isolated by Dr. Lyda would stand the very slight stresses to which it is exposed in the Ussing chamber.

With the simple filtration method, however, some valid information has been obtained, and we can already say that this is a membrane which has very considerable permeability. It seems premature to draw any far-reaching conclusions at this point.

There are two questions that I would like to ask Dr. Lyda. Did any of his experiments suggest anything in the way of a directional permeability? In other words, does this membrane work equally well or equally badly for globulin in the direction from the choroid to the retina as it does in the opposite direction?

Another question that came to my mind as I looked at the pH curve was this: Are the changes that you produced by running through the membrane fluids with low pH reversible? Does the membrane, after you have run through it a fluid of pH of 3.5, return to more or less the original condition if you wash out that very acid solution and go back to just the saline?

I believe Dr. Lyda and his group are to be commended on having conceived an original and promising idea and on having done quite a bit of valuable groundwork in the pursuit of that idea.

DR. WOOD LYDA (closing): Dr. Kronfeld's query as to any possible differences in flow rates between membranes with the choroid facing the reservoir instead of Bruch's membrane is an interesting one.

In the early portions of the experiments a membrane was sometimes inadvertently placed so that the choroid side of the membrane did face the reservoir. Interestingly enough, the 10 or so times it did happen the flow rate curves were essentially the same as were found with the membrane properly placed.

There were too few of these membranes to draw any definite conclusions but we felt that the similarity of the curves indicated that the small amount of choroid remaining attached to the membrane did not invalidate the comparative data.

As far as the irreversible effects of changes in pH values is concerned, we found in pilot studies that in pH over 10 and under 2.0, the flow rate would not return to a normal control value. These extremes produced irreversible changes in the membrane. It was for this reason that we used a range of pH from 8.9 to 3.9 to study the pH effect. I would like to point out in passing that Bruch's membrane is composed of many protein complexes with many iso-electric points. We believe that our

finding of an iso-electric point between pH 4.7 to 5.2 only represents a summation of the various points actually in operation. We feel that this may also be true of Friedenwald's iso-electric point of pH 5.47 for the ciliary body boundary.

We wish to thank Dr. Kronfeld for the kindness of his discussion for we feel that there are many objections to the experimental procedures we employedfi of which some we are aware and some not. However, from these crude experiments we are convinced that the lamina vitrea is involved in a physiologic function that is worthy of serious and advanced investigation.

# THE EFFECTS OF A GANGLIONIC BLOCKING DRUG (PENTAPYRROLIDINIUM)*

## On aqueous humor flow of normal and glaucomatous eyes

David A. Rosen, M.D.
*Kingston, Ontario*

### Introduction

Work has been presented in the past indicating that the administration of the ganglionic blocking drug pentapyrrolidinium (pentolinium, Ansolysen) produces in normal subjects striking alterations of certain features of aqueous humor flow.[1] Among these effects are:

1. A prolonged inhibition of aqueous flow averaging at the maximum 77.07 percent.
2. A simultaneous decrease in facility of aqueous outflow.
3. A fall of intraocular pressure of 12.55 percent.
4. An inhibition of the consensual fall in intraocular pressure during tonography.

The present report deals with the effects of pentolinium on aqueous flow in subjects with open-angle glaucoma. The data were obtained from tonographic recordings made with a Mueller electronic tonometer attached to a Leeds and Northrup Model B Speedomax Recorder. Friedenwald's 1954 tables,[2] Grant's equations,[3] and Linnèr's value of 11 mm. Hg for P $v$[4] were employed in the calculations.

### Results

#### 1. The effect of pentolinium in wide-angle glaucoma

Ten patients with open-angle glaucoma were selected for study. The studies were carried out shortly after the diagnosis of glaucoma was first established and before therapy had been started. None of the eyes had been subjected to surgery and in no instance was the visual field loss extensive.

In these subjects a control tonographic record was made on both eyes. The patients were then given 20 mg. of pentolinium by mouth and were permitted to remain in a recumbent position. The blood pressure fall under these circumstances was inconstant and small, never exceeding 14 mm. Hg systolic or diastolic in these and the subsequent studies. One hour after the ingestion of the drug a second tonographic record was made.

A mean decline of intraocular pressure of 6.4 percent occurred in the right eye and of 5.7 percent in the left. The coefficient of outflow facility did not change appreciably. Table 1 contains the aqueous flow rates before and after the administration of the drug. The mean fall in aqueous flow in the right eye was 18.3 percent and in the left eye was 17.8 percent. This degree of inhibition of flow is much smaller than that previously noted in normal eyes.[1]

Each of the patients was then placed on a miotic and was re-examined anywhere from three days to two weeks later in a similar manner. The intraocular pressure had fallen from a premiotic mean of 42.5 mm. Hg and 44.8 mm. Hg for the right and left eye respectively to 29.0 and 30.0 mm. Hg. One hour after pentolinium administration the

* From the Department of Ophthalmology, Queen's University. This work was supported by Grant M.P. 519 of the National Research Council of Canada.

TABLE 1

EFFECT OF PENTOLINIUM ON AQUEOUS FLOW IN UNTREATED CHRONIC SIMPLE GLAUCOMA

| Subject | Control | | Pentolinium 20 mg. | | | |
|---|---|---|---|---|---|---|
| | Right cu.mm./min. | Left cu.mm./min. | Right cu.mm./min. | Δ% | Left cu.mm./.min. | Δ% |
| J.J. | 0.85 | 0.55 | 0.82 | − 3.5 | 0.50 | − 9.1 |
| B.B. | 1.46 | 1.32 | 1.40 | − 4.1 | 1.16 | −12.1 |
| M.A. | 3.64 | 3.46 | 3.22 | −11.5 | 3.08 | −11.0 |
| W.C. | 2.66 | 2.84 | 2.12 | −20.3 | 2.08 | −26.8 |
| C.C. | 1.86 | 2.02 | 1.46 | −21.5 | 1.57 | −22.3 |
| C.G. | 2.12 | 2.46 | 1.86 | −12.3 | 2.10 | −14.6 |
| K.N. | 4.08 | 3.86 | 3.55 | −13.0 | 3.07 | −20.5 |
| M.L. | 1.15 | 1.08 | 0.66 | −42.6 | 0.87 | −19.4 |
| J.K. | 3.12 | 2.64 | 2.28 | −26.9 | 2.14 | −18.9 |
| J.D. | 2.79 | 2.97 | 2.04 | −26.9 | 2.28 | −23.2 |
| Mean | 2.37 | 2.32 | 1.94 | −18.3 | 1.79 | −17.8 |
| S.D. | ±1.08 | ±1.07 | ±0.94 | ±11.9 | ±1.07 | ± 5.83 |
| S.E.M. | ±0.34 | ±0.34 | ±0.30 | ± 3.8 | ±0.34 | ± 1.84 |
| P. | | | | < 0.01 | | < 0.01 |

pressures had fallen 13.6 percent to 25.0 mm. Hg, on the right, and 10.2 percent to 26.3 mm. Hg on the left. Concomitantly, the outflow coefficient diminished by 13.3 percent on the right and 23.0 percent on the left.

The values calculated for aqueous flow are given In Table 2. The inhibition of flow produced by pentolinium was increased to a mean of 36.2 percent in the right eye and 37.2 percent in the left eye.

The difference in aqueous flow inhibition produced by pentolinium before and after the use of miotics was found to be well within the range of statistical significance (table 3).

### 2. THE EFFECT OF PILOCARPINE ON AQUEOUS FLOW INHIBITION OF PENTOLINIUM IN NORMAL EYES

The accentuation of the inhibitory effect of pentolinium on aqueous flow by miotics suggested a similar investigation on normal eyes. Eight patients who had no organic ocular disease were investigated in the following manner:

After a control tonographic tracing had

TABLE 2

EFFECTS OF PENTOLINIUM ON AQUEOUS FLOW IN TREATED CHRONIC SIMPLE GLAUCOMA

| Subject | Treatment | Control | | Pentolinium 20 mg. | | | |
|---|---|---|---|---|---|---|---|
| | | Right cu.mm./min. | Left cu.mm./min. | Right cu.mm./ min. | Δ% | Left cu.mm./ min. | Δ% |
| J.J. | Pilocarpine 2% t.i.d. | 0.64 | 0.47 | 0.51 | −20.3 | 0.33 | −29.8 |
| B.B. | Doryl 1% q.i.d. | 1.84 | 1.38 | 0.96 | −47.8 | 0.88 | −36.2 |
| M.A. | Eserine ¼% t.i.d. | 3.22 | 3.86 | 2.90 | − 9.9 | 3.02 | −21.8 |
| W.G. | Pilocarpine 1% b.i.d. | 2.84 | 3.12 | 1.62 | −42.9 | 1.24 | −60.3 |
| C.C. | Pilocarpine 1% b.i.d. | 2.22 | 1.84 | 1.44 | −35.1 | 1.35 | −26.6 |
| C.B. | Doryl 1% t.i.d. | 2.05 | 2.22 | 1.12 | −45.4 | 1.00 | −54.9 |
| K.N. | Pilocarpine 1% t.i.d. | 3.66 | 3.45 | 2.33 | −36.3 | 2.12 | −38.6 |
| M.L. | Pilocarpine 1% b.i.d. | 1.67 | 1.34 | 0.88 | −47.3 | 0.63 | −53.0 |
| J.K. | Pilocarpine 1% b.i.d. | 4.64 | 2.86 | 2.84 | −38.8 | 2.48 | −13.3 |
| J.D. | Pilocarpine 2% t.i.d. | 3.02 | 2.67 | 1.86 | −38.4 | 1.67 | −37.4 |
| Mean | | 2.58 | 2.32 | 1.65 | −36.2 | 1.47 | −37.2 |
| S.D. | | ±1.14 | ±1.07 | ±0.83 | ±12.2 | ±0.85 | ±15.2 |
| S.E.M. | | ±0.36 | ±0.34 | ±0.26 | ± 3.86 | ±0.27 | ± 4.80 |
| P. | | | | | < 0.01 | | < 0.01 |

TABLE 3

COMPARISON OF AQUEOUS FLOW INHIBITION BY PENTOLINIUM IN CHRONIC SIMPLE GLAUCOMA BEFORE AND AFTER MIOTICS

| | Right Δ% | | Left Δ% | |
|---|---|---|---|---|
| | Before (1) | After (2) | Before (1) | After (2) |
| Mean | −18.3 | −36.2 | −17.8 | −37.2 |
| S.D. | ±11.9 | ±12.2 | ± 5.83 | ±15.2 |
| S.E.M. | ± 3.8 | ± 3.86 | ± 1.84 | ± 4.80 |
| P. | < 0.01 | < 0.01 | < 0.01 | < 0.01 |
| P 1, 2 | <0.01 | | <0.01 | |

been made on both eyes the patients were given 20 mg. of pentolinium by mouth. One hour later a second recording was made. The intraocular pressure was found to have diminished 8.2 percent on the right and 9.5 percent on the left, while the mean fall in aqueous flow rate was 59.3 percent in the right eye and 29.7 percent in the left eye (table 4).

On the following day each of the subjects received pilocarpine (one percent) three times daily in the right eye only. One day later the miotic was again instilled in the right eye at 8:00 A.M. and starting at 9:00 A.M. two tonographic recordings were made, separated by one hour and by the administration of a further oral dose of pentolinium.

In this experiment pentolinium produced a fall in intraocular pressure of 9.8 percent on the right and of 13.7 percent on the left. The mean drops in aqueous flow rates were 21.2 percent and 32.2 percent in the right and left eyes respectively (table 5). The right eye records of two subjects (No. 3 and No. 4) were inadmissible because of faulty tracings.

While the results obtained suggest an interference with the flow-inhibiting effect of pentolinium by pilocarpine in normal eyes they lack statistical significance (table 6). It will be necessary to repeat this study on a larger number of patients.

## Discussion

The explanation for these phenomena can only be a matter of conjecture. One could more readily explain the possible interference with the effect of pentolinium by pilocarpine in normal subjects than the significant enhancement of its effect in patients with open-angle glaucoma. It has been found, for example,[5] that the administration of pilocarpine by mouth will abolish the mouth dryness, eye dryness and the constipation produced in humans by various ganglionic blocking drugs.

Perhaps stimulation by a cholinergic agent such as pilocarpine renders parasympathetically innervated structures relatively refractory to impulses normally passing along post-

TABLE 4

EFFECT OF PENTOLINIUM ON AQUEOUS FLOW IN NORMAL EYES BEFORE ADMINISTRATION OF PILOCARPINE

| Subject | Control | | Pentolinium 20 mg. | | | |
|---|---|---|---|---|---|---|
| | Right cu.mm./min. | Left cu.mm./min. | Right cu.mm./min. | Δ% | Left cu.mm./min. | Δ% |
| 1 | 1.46 | 1.71 | 0.54 | −63.0 | 0.59 | −65.5 |
| 2 | 1.57 | 1.10 | 0.63 | −59.9 | 0.44 | −60.0 |
| 3 | 1.36 | 1.61 | −0.63 | −146.3 | −0.38 | −123.6 |
| 4 | 1.53 | 1.91 | 0.53 | −65.4 | 1.00 | −47.6 |
| 5 | 4.14 | 0.73 | 0.74 | −82.1 | 0.81 | +10.9 |
| 6 | 2.47 | 1.14 | 1.08 | −56.3 | 0.28 | −75.4 |
| 7 | 2.57 | 1.32 | 1.26 | −51.0 | 1.50 | +13.6 |
| 8 | 1.90 | 1.13 | 2.84 | +49.5 | 2.37 | +10.97 |
| Mean | 2.13 | 1.33 | 0.87 | −59.3 | 0.83 | −29.74 |
| S.D. | ±0.93 | ±1.23 | ±0.97 | ±53.5 | ±0.83 | ±72.05 |
| S.E.M. | ±0.33 | ±0.43 | ±0.34 | ±18.9 | ±0.29 | ±25.47 |
| P. | | | | 0.017 | | 0.26 |

TABLE 5

AFFECT OF PENTOLINIUM ON AQUEOUS FLOW IN NORMAL EYES AFTER ADMINISTRATION OF PILOCARPINE IN RIGHT EYE ONLY, THREE TIMES DAILY, ON DAY BEFORE AND ONE HOUR BEFORE TEST

| Subject | Control | | Pentolinium 20 mg. | | | |
|---|---|---|---|---|---|---|
| | Right cu. mm./min. | Left | Right cu.mm./min. | Δ% | Left cu.mm./min. | Δ% |
| 1 | 1.03 | 0.89 | 1.02 | − 1.0 | 0.63 | −29.2 |
| 2 | 1.68 | 2.38 | 1.84 | + 9.5 | 1.03 | −56.7 |
| 3 | | 1.44 | | | 0.41 | −71.5 |
| 4 | | 1.92 | | | 0.39 | −80.2 |
| 5 | 0.53 | 3.82 | 0.61 | +15.1 | 3.05 | −20.1 |
| 6 | 1.52 | 2.11 | 1.08 | −28.9 | 1.45 | −31.3 |
| 7 | 2.81 | 1.57 | 2.58 | −81.9 | 2.83 | +80.3 |
| 8 | 1.88 | 1.76 | 1.13 | −39.9 | 0.90 | −48.9 |
| Mean | 1.58 | 1.99 | 1.03 | −21.2 | 1.34 | −32.2 |
| S.D. | ±0.78 | ±0.87 | ±0.71 | ±36.76 | ±1.05 | ±50.0 |
| S.E.M. | ±0.32 | ±0.31 | ±0.29 | ±15.0 | ±0.37 | ±17.7 |
| P. | | | | 0.21 | | 0.11 |

ganglionic nerves. Diminishing the number of postganglionic impulses by ganglionic blockade would in such a state have a lessened influence upon the aqueous secretion by the ciliary body.

It is more difficult, however, to arrive at an explanation for the enhancement of the effect of pentolinium through the administration of miotics to patients with open-angle glaucoma. It may be that in the resting state the glaucomatous eye receives a subnormal quality of autonomic innervation. The miotic drug may heighten the "innervational tone" of the autonomic nerves supplying the eye. Ganglionic blockade under these circumstances might be expected to produce a greater inhibition of aqueous secretion and flow.

Whatever their explanation these observations indicate a difference in the neurophysiologic make-up of the eyes affected by open-angle glaucoma. This difference may in some way be significant in the pathogenesis of the disease.

*Queen's University.*

TABLE 6

COMPARISON OF AQUEOUS FLOW INHIBITION BY PENTOLINIUM IN NORMAL EYES BEFORE AND AFTER PILOCARPINE IN RIGHT EYE ONLY

| | Right Δ% | | Left Δ% | |
|---|---|---|---|---|
| | Before Pilocarpine (1) | After Pilocarpine (2) | No Pilocarpine (1) | No Pilocarpine (2) |
| Mean | −59.3 | −21.2 | −29.74 | −32.2 |
| S.D. | ±53.5 | ±36.76 | ±72.05 | ±50.0 |
| S.E.M. | ±18.9 | ±15.0 | ±25.47 | ±17.7 |
| P. | 0.017 | 0.21 | 0.26 | 0.11 |
| P 1, 2 | 0.15 | | >0.5 | |

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TABLE 3

COMPARISON OF AQUEOUS FLOW INHIBITION BY PENTOLINIUM IN CHRONIC SIMPLE GLAUCOMA BEFORE AND AFTER MIOTICS

| | Right Δ% | | Left Δ% | |
|---|---|---|---|---|
| | Before (1) | After (2) | Before (1) | After (2) |
| Mean | −18.3 | −36.2 | −17.8 | −37.2 |
| S.D. | ±11.9 | ±12.2 | ± 5.83 | ±15.2 |
| S.E.M. | ± 3.8 | ± 3.86 | ± 1.84 | ± 4.80 |
| P. | < 0.01 | < 0.01 | < 0.01 | < 0.01 |
| P 1, 2 | <0.01 | | <0.01 | |

been made on both eyes the patients were given 20 mg. of pentolinium by mouth. One hour later a second recording was made. The intraocular pressure was found to have diminished 8.2 percent on the right and 9.5 percent on the left, while the mean fall in aqueous flow rate was 59.3 percent in the right eye and 29.7 percent in the left eye (table 4).

On the following day each of the subjects received pilocarpine (one percent) three times daily in the right eye only. One day later the miotic was again instilled in the right eye at 8:00 A.M. and starting at 9:00 A.M. two tonographic recordings were made, separated by one hour and by the administration of a further oral dose of pentolinium.

In this experiment pentolinium produced a fall in intraocular pressure of 9.8 percent on the right and of 13.7 percent on the left. The mean drops in aqueous flow rates were 21.2 percent and 32.2 percent in the right and left eyes respectively (table 5). The right eye records of two subjects (No. 3 and No. 4) were inadmissible because of faulty tracings.

While the results obtained suggest an interference with the flow-inhibiting effect of pentolinium by pilocarpine in normal eyes they lack statistical significance (table 6). It will be necessary to repeat this study on a larger number of patients.

## DISCUSSION

The explanation for these phenomena can only be a matter of conjecture. One could more readily explain the possible interference with the effect of pentolinium by pilocarpine in normal subjects than the significant enhancement of its effect in patients with open-angle glaucoma. It has been found, for example,[5] that the administration of pilocarpine by mouth will abolish the mouth dryness, eye dryness and the constipation produced in humans by various ganglionic blocking drugs.

Perhaps stimulation by a cholinergic agent such as pilocarpine renders parasympathetically innervated structures relatively refractory to impulses normally passing along post-

TABLE 4

EFFECT OF PENTOLINIUM ON AQUEOUS FLOW IN NORMAL EYES BEFORE ADMINISTRATION OF PILOCARPINE

| Subject | Control | | Pentolinium 20 mg. | | | |
|---|---|---|---|---|---|---|
| | Right cu.mm./min. | Left cu.mm./min. | Right cu.mm./min. | Δ% | Left cu.mm./min. | Δ% |
| 1 | 1.46 | 1.71 | 0.54 | −63.0 | 0.59 | −65.5 |
| 2 | 1.57 | 1.10 | 0.63 | −59.9 | 0.44 | −60.0 |
| 3 | 1.36 | 1.61 | −0.63 | −146.3 | −0.38 | −123.6 |
| 4 | 1.53 | 1.91 | 0.53 | −65.4 | 1.00 | −47.6 |
| 5 | 4.14 | 0.73 | 0.74 | −82.1 | 0.81 | +10.9 |
| 6 | 2.47 | 1.14 | 1.08 | −56.3 | 0.28 | −75.4 |
| 7 | 2.57 | 1.32 | 1.26 | −51.0 | 1.50 | +13.6 |
| 8 | 1.90 | 1.13 | 2.84 | +49.5 | 2.37 | +10.97 |
| Mean | 2.13 | 1.33 | 0.87 | −59.3 | 0.83 | −29.74 |
| S.D. | ±0.93 | ±1.23 | ±0.97 | ±53.5 | ±0.83 | ±72.05 |
| S.E.M. | ±0.33 | ±0.43 | ±0.34 | ±18.9 | ±0.29 | ±25.47 |
| P. | | | | 0.017 | | 0.26 |

TABLE 5

AFFECT OF PENTOLINIUM ON AQUEOUS FLOW IN NORMAL EYES AFTER ADMINISTRATION OF PILOCARPINE IN RIGHT EYE ONLY, THREE TIMES DAILY, ON DAY BEFORE AND ONE HOUR BEFORE TEST

| Subject | Control | | Pentolinium 20 mg. | | | |
|---|---|---|---|---|---|---|
| | Right cu. mm./min. | Left | Right cu.mm./min. | Δ% | Left cu.mm./min. | Δ% |
| 1 | 1.03 | 0.89 | 1.02 | − 1.0 | 0.63 | −29.2 |
| 2 | 1.68 | 2.38 | 1.84 | + 9.5 | 1.03 | −56.7 |
| 3 | | 1.44 | | | 0.41 | −71.5 |
| 4 | | 1.92 | | | 0.39 | −80.2 |
| 5 | 0.53 | 3.82 | 0.61 | +15.1 | 3.05 | −20.1 |
| 6 | 1.52 | 2.11 | 1.08 | −28.9 | 1.45 | −31.3 |
| 7 | 2.81 | 1.57 | 2.58 | −81.9 | 2.83 | +80.3 |
| 8 | 1.88 | 1.76 | 1.13 | −39.9 | 0.90 | −48.9 |
| Mean | 1.58 | 1.99 | 1.03 | −21.2 | 1.34 | −32.2 |
| S.D. | ±0.78 | ±0.87 | ±0.71 | ±36.76 | ±1.05 | ±50.0 |
| S.E.M. | ±0.32 | ±0.31 | ±0.29 | ±15.0 | ±0.37 | ±17.7 |
| P. | | | | 0.21 | | 0.11 |

ganglionic nerves. Diminishing the number of postganglionic impulses by ganglionic blockade would in such a state have a lessened influence upon the aqueous secretion by the ciliary body.

It is more difficult, however, to arrive at an explanation for the enhancement of the effect of pentolinium through the administration of miotics to patients with open-angle glaucoma. It may be that in the resting state the glaucomatous eye receives a subnormal quality of autonomic innervation. The miotic drug may heighten the "innervational tone" of the autonomic nerves supplying the eye. Ganglionic blockade under these circumstances might be expected to produce a greater inhibition of aqueous secretion and flow.

Whatever their explanation these observations indicate a difference in the neurophysiologic make-up of the eyes affected by open-angle glaucoma. This difference may in some way be significant in the pathogenesis of the disease.

*Queen's University.*

TABLE 6

COMPARISON OF AQUEOUS FLOW INHIBITION BY PENTOLINIUM IN NORMAL EYES BEFORE AND AFTER PILOCARPINE IN RIGHT EYE ONLY

| | Right Δ% | | Left Δ% | |
|---|---|---|---|---|
| | Before Pilocarpine (1) | After Pilocarpine (2) | No Pilocarpine (1) | No Pilocarpine (2) |
| Mean | −59.3 | −21.2 | −29.74 | −32.2 |
| S.D. | ±53.5 | ±36.76 | ±72.05 | ±50.0 |
| S.E.M. | ±18.9 | ±15.0 | ±25.47 | ±17.7 |
| P. | 0.017 | 0.21 | 0.26 | 0.11 |
| P 1, 2 | 0.15 | | >0.5 | |

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## Discussion

Dr. Elmer J. Ballintine (Cleveland): Dr. Rosen has shown that the tonograms of normal and glaucomatous eyes have steeper slopes and lower initial scale readings than tonograms of the same eyes obtained one hour after administration of pentapyrrolidinium. These changes seem to indicate that the drug reduced the production of aqueous humor.

The reduction was accompanied by a decrease of the coefficient of outflow so that the resulting reduction of intraocular pressure was inconspicuous and insignificant. The reduction of flow appeared to be more marked in glaucomatous eyes treated with miotics than in untreated glaucomatous or normal eyes.

If this interpretation is true, pentapyrrolidinium is unique among drugs known to decrease production of aqueous humor, in that the compensatory decrease of C occurs so rapidly, especially in glaucomatous eyes. Before this uniqueness is accepted, alternative explanations of the data should be considered.

The data which exhibit the largest apparent differences in flow are those which show the effect of pentapyrrolidinium administered to glaucomatous patients under treatment with miotics. These data (some taken from raw data furnished by Dr. Rosen) can be approximately summarized as follows:

| | Average $P_o$ | Average C |
|---|---|---|
| Before drug | 29.5 | 0.14 |
| After drug | 25.6 | 0.115 |

If the 5.5-gm. weight had been used, the average results of measurement of the tonogram would have been:

| | $R_1$ | $R_2$ |
|---|---|---|
| Before drug | 1.75 | 3.4 |
| After drug | 2.6 | 4.1 |

Assume that the drug affects scleral rigidity only, that the true C and true F are unchanged, and that scleral rigidity was normal before the drug was given. In the steady-state, $P_o$ would be the same as before administration of the drug, but the new scleral rigidity is found from the slope of the line on the scleral rigidity nomograph passing through $P_o = 29.5$ and $R_1 = 2.6$ and is approximately 0.018.

In other words, the true $P_o$ before and after the drug was 29.5 because neither C nor F changed. In that case, the new value for scleral rigidity can be obtained from a scleral rigidity nomograph by drawing a line through $P_o$ and $R_1$ after administration of the drug, and the slope of that line is 0.018, or the new scleral rigidity, instead of being 0.0215, is now 0.018. If you follow through the calculation of C for R.1 and R.2 after administration of the drug, the C turns out to be 0.26.

Precisely similar results are obtained if it is assumed that the 7.5-gm. weight was used for tonography and the analysis is similar. Thus, it is possible to postulate that the drug, by reducing scleral rigidity only moderately, could produce the observed results. These considerations emphasize the fact that tonography alone does not distinguish between changes in scleral rigidity and changes in flow or in C.

Although pentapyrrolidinium is said to have no effect on skeletal muscle tone, such blocking agents may have anomalous effects on extraocular muscles, and the drug might produce decreased scleral rigidity by decreasing the tone of the extraocular muscles.

Because of the implications of this work in relation to the mechanisms controlling C, I would like to urge further efforts to elucidate the mechanism of the ocular effects of the drug.

I want to commend Dr. Rosen on the thoroughness of his observations. We have attempted to carry out somewhat similar observations, and have encountered great difficulties in persuading these patients to co-operate, especially the normal patients, in a series of tonograms at different times of the day and to use the medications properly. The amount of work involved is enormous, and the care with which it has been carried out is to be complimented.

Dr. David A. Rosen (closing): I wish to thank Dr. Ballintine for his very searching analysis of the data which I presented to him.

I am particularly intrigued by his suggestion of an altered scleral rigidity to account for these results, and it will be our purpose to look into this factor. Unhappily we are not set up as is Dr. Breinin and some other people to measure electrical activity in extraocular muscles.

I am also grateful that he emphasized the fact that one must be extremely cautious in assigning too much mathematical significance to tonographic observations. I think that is a point which he made particularly well, and which bears careful consideration.

# THE STEADY-STATE TURNOVER OF TRITIATED WATER*

## IN THE ANTERIOR CHAMBER OF RABBITS

MAX FRIEDMAN, A.B., FRANK W. NEWELL, M.D., GEORGE V. LEROY, M.D., AND GEORGE T. OKITA, M.D.
*Chicago, Illinois*

The steady-state rate of exchange of water between the anterior aqueous humor and the blood has been determined only by Kinsey, Grant, and Cogan[1] These workers used deuterium oxide ($D_2O$) as a tracer substance for water and reported a half-life of the anterior aqueous humor of 2.7 minutes when measured from aqueous to blood and less than 5.4 minutes in the direction of blood to aqueous.

The development of liquid scintillation counting[2,3] for the assay of tritium has facilitated the analysis of small amounts of radioactivity and permitted the use of minute amounts of tritiated water (HTO) as a tracer substance for water. Tritium is a beta emitter with the following decay:

$$1H^3 \quad 2He^3 + \beta$$

(Av. energy 5.7 Kev)

It has a half-life of 12.4 years.[4,5]

### PROCEDURE

A tracer dose of HTO of approximately 1.0 mc. was diluted with isotonic saline to 1.0 ml. and injected intraperitoneally in an anesthetized male albino rabbit. Serial samples of femoral artery blood were collected in heparinized tubes, centrifuged and the supernatant fluid removed. Paracentesis of the anterior chamber was done at varying periods, in different animals, after the administration of the HTO. The arterial plasma and the aqueous humor samples were then assayed for HTO activity in a liquid scintillation counter in a toluene system using 2,5 diphenyl-oxazole (DPO) as the phosphor. The samples were prepared for counting by mixing 0.1 ml. of the fluid to be assayed with 5.0 ml. of absolute ethanol in a standard weighing bottle, and adding 50 ml. of toluene and 0.1 gm. of DPO. The samples were precooled to −17°C. prior to analysis. Preliminary studies indicated that no significant error was introduced by regarding all tritium activity as occurring in the water fraction of the plasma exclusively.

### THEORETIC CONSIDERATIONS

The steady-state rate of exchange of water between the plasma and anterior chamber aqueous humor was estimated by solving for $k_{pa}$ by numerical integration of the differential equation:

$$\frac{dCa(t)}{dt} = K_{pa}\,[C_p(t) - Ca\,(t)] \qquad \text{(Equation 1)}$$

where:

$Ca(t)$ = activity of HTO in the anterior chamber aqueous humor at time t

$Cp(t)$ = activity of HTO in arterial plasma at time t

$k_{pa}$ = the fraction of anterior chamber aqueous humor water which exchanges with the blood plasma each minute.

The corresponding half-life of the anterior chamber aqueous humor water is then determined by the formula:

$$T^{1/2} = \frac{\log_e 2}{k_{pa}} \qquad \text{(Equation 2)}$$

Posterior aqueous was not removed for analysis. Thus it was not possible to derive values for $k_f$ and $K_d$ using the equation of Kinsey and Palm[6] and Friedenwald and Becker:[7]

$$\frac{dCa}{dt} = k_f\,(C - Ca) + k_d\,(Cp - Ca)$$

### RESULTS

The experiment was performed on 23

* From the Department of Surgery (Ophthalmology), The University of Chicago. This study was supported in part by Fight for Sight Grant No. 103 of the National Council to Combat Blindness.

TABLE 1

RABBIT 10, SERIES A, WEIGHT—2.20 KG.

| Time (min.) | Net HTO Activity | |
|---|---|---|
| | Arterial Plasma | Aqueous Humor |
| 0 | 0 | 0 |
| 1 | 15116 | — |
| 2 | 21624 | 2525 (R.E.) |
| 5 | 22163 | — |
| 10 | 20625 | 11725 (L.E.) |
| 15 | 18925 | — |
| 20 | 18160 | — |

rabbits. In series A the HTO was administered intraperitoneally and in series B intravenously. The data concerning Rabbit 10 of series A (table 1) is used as an example of the calculation.

Three points were determined on the aqueous humor curve, the origin and the concentration of tritium for each eye (fig. 1). Numerical integration of equation 1 was performed on the interval [0,2] and on the interval [2,10] providing two solutions for $k_{pa}$.

$$k_{pa}\ [0,2] = 0.108$$
$$k_{pa}\ [2,10] = 0.080$$

By varying the times at which samples of aqueous humor were taken the values of $k_{pa}$ were determined for several different time intervals (table 2). Thirty-three values of $k_{pa}$ were calculated. The average was 0.094 corresponding to a half-life of 7.4 minutes (equation 2). The minimum value was 0.052 corresponding to a half-life of 13.3 minutes and the maximum value was 0.156 cor-

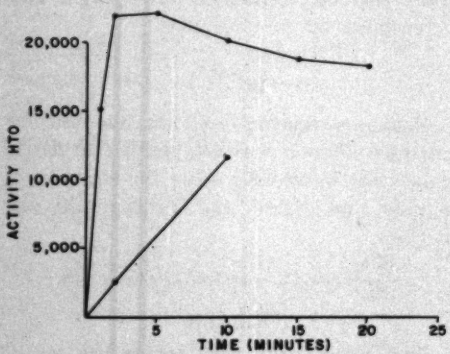


Fig. 1 (Friedman, Newell, LeRoy and Okita). Graph of data obtained in Animal 10.

TABLE 2

SERIES A. RABBITS WERE GIVEN ONE MC. HTO INTRAPERITONEALLY. THE CALCULATED VALUES OF $k_{pa}$ ARE SHOWN WITH CORRESPONDING TIME INTERVALS

| Rabbit | Weight (kgm.) | Interval (min.) | $k_{pa}$ |
|---|---|---|---|
| 1 | 3.7 | [0, 2] | 0.135 |
| | | [2, 8] | 0.099 |
| 2 | 3.9 | [0, 2] | 0.153 |
| | | [2, 6] | 0.117 |
| 3 | 3.2 | [0, 4] | 0.058 |
| | | [4, 9] | 0.055 |
| 4 | 4.3 | [0, 10] | 0.062 |
| | | [10, 20] | 0.134 |
| 5 | 3.6 | [0, 5] | 0.102 |
| | | — | — |
| 6 | 3.1 | [0, 5] | 0.058 |
| | | [5, 15] | 0.080 |
| 7 | 4.3 | [0, 5] | 0.096 |
| | | [5, 15] | 0.086 |
| 8 | 2.6 | [0, 5] | 0.052 |
| | | [5, 15] | 0.056 |
| 9 | 2.7 | [0, 10] | 0.057 |
| | | [10, 20] | 0.062 |
| 10 | 2.2 | [0, 2] | 0.108 |
| | | [2, 10] | 0.080 |
| 11 | 4.5 | [0, 2] | 0.156 |
| | | [2, 10] | 0.095 |
| 12 | 2.3 | [0, 10] | 0.071 |
| | | — | — |
| 13 | 3.8 | [0, 15] | 0.069 |
| | | — | — |
| 14 | 4.1 | [0, 15] | 0.067 |
| | | — | — |
| 15 | 3.6 | [0, 10] | 0.114 |
| | | — | — |

TABLE 3

SERIES B. RABBITS WERE GIVEN 0.1 MC. HTO INTRAVENOUSLY. ALL VALUES OF $k_{pa}$ ARE CALCULATED FOR THE TIME INTERVAL [2, 5] MINUTES

| Rabbit | Weight (kg.) | Interval (min.) | $k_{pa}$ |
|---|---|---|---|
| 1 | .7 | [2, 5] | 0.087 |
| 2 | 2.7 | [2, 5] | 0.073 |
| 3 | 4.1 | [2, 5] | 0.126 |
| 4 | 4.5 | [2, 5] | 0.110 |
| 5 | 4.6 | [2, 5] | 0.080 |
| 6 | 3.8 | [2, 5] | 0.134 |
| 7 | 3.4 | [2, 5] | 0.113 |
| 8 | 3.8 | [2, 5] | 0.154 |

responding to a half-life of 4.4 minutes.

The results in the present series show a great variation and the calculated rate of turnover per minute ranges from 5.2 percent to 15.6 percent. It should be noted that generally the values of $k_{pa}$ tend to decrease as numerical integration is carried out on the later time intervals. A similar tendency was noted by Palm[8] in studying the rate of penetration of ethyl alcohol into the anterior chamber aqueous. He suggested at that time that diffusion within the anterior chamber was a significant factor which was not considered in simplified mathematical formulations. We believe that failure to account for mixing time within the anterior chamber is an important cause of this apparent decrease in the value of $k_{pa}$ with time.

## DISCUSSION

The measurement of the steady-state of a rapidly moving molecule such as water involves a number of factors which give rise to many variations in the rate of exchange in different animals. The water in the blood is undergoing rapid equilibration with all other body fluids and in the methods used here the level of plasma water varied markedly with time. General anesthesia introduces variables relating to blood pressure and blood flow to various organs so that the results from animal to animal are not strictly comparable. However, it should be remarked that factors such as excitement and activity in animals not anesthetized might exert similar effects.

TABLE 4

AVERAGE $k_{pa}$ CALCULATED FOR SEVERAL DIFFERENT TIME INTERVALS

| Interval | No. of Values | Range | Average $k_{pa}$ | Half-Life (min.) |
|---|---|---|---|---|
| [0, 2] | 4 | 0.108–0.156 | 0.138 | 5.0 |
| [0, 5] | 3 | 0.058–0.102 | 0.085 | 8.2 |
| [2, 5] | 8 | 0.073–0.154 | 0.110 | 6.3 |
| [2, 10] | 3 | 0.071–0.095 | 0.081 | 8.6 |
| [0, 15] | 2 | 0.067–0.069 | 0.068 | 10.0 |
| [5, 15] | 3 | 0.056–0.086 | 0.074 | 9.4 |
| [10, 20] | 2 | 0.062–0.134 | 0.098 | 7.1 |

In this study femoral arterial blood was used as reflecting the concentration of the isotope in the plasma. This, of course, did not necessarily indicate the concentration of HTO in the capillaries of the eye where the exchange, in which we were interested, was taking place. Unquestionably too, the blood flow through the ocular capillaries is one of the most, if not the most important factor, governing the exchange of HTO between the plasma and the eye and this flow cannot be measured, accurately.

The mixing of HTO in the plasma and in the eye is again an obviously important and unmeasured factor. We are reasonably certain from other studies that the HTO is dispersed through the water phase of the plasma and is entirely available for exchange with the eye and other body fluids. We do not know however how completely the HTO is mixed with this water to be available for exchange. Within the anterior aqueous it seems most likely that the concentration of HTO would be greatest near the surface of the iris where the exchange is taking place and lowest near the cornea. Thus the sample of aqueous reflects inadequate mixing with the value of $k_{pa}$ lower than is actually true.

In addition there is an establishment of equilibrium through diffusional exchange of water between the various structures of the eye (fig. 2) the values of which are not reflected in Equation 1. Thus a portion of the HTO which enters the anterior aqueous from the blood escapes to other intraocular structures and this tends to make the values of $k_{pa}$ decrease for later time intervals. The

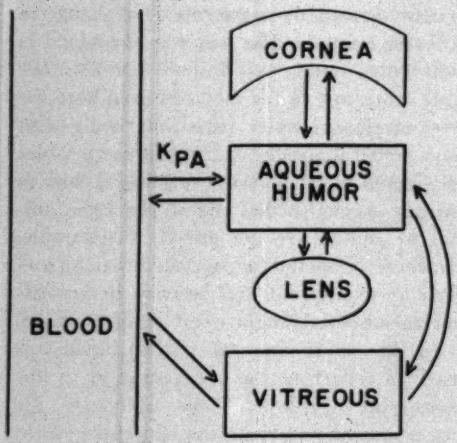


Fig. 2 (Friedman, Newell, LeRoy and Okita). The exchange of HTO within the intraocular structures.

assumption that the exchange in two eyes of the same rabbit are similar may well be invalid since paracentesis may lead to changes in the dynamics of the contralateral eye.

A possible error is that HTO, a heavier molecule than ordinary water behaves differently than ordinary water in a biologic system. Other studies have shown HTO to be toxic to unicellular animals but it is generally believed that in a diffusional process it accurately reflects water movement.

The results of Kinsey, Grant and Cogan[1] who used deuterium oxide are not comparable, in many respects, to the values reported here. In four animals the deuterium oxide was administered intraperitoneally and aqueous humor samples removed at varying periods thereafter. Maximum half-life values were estimated to be less than 4.25, 7.2, 6.0, and 4.0 minutes in each animal, values similar to those found by us. In other rabbits aqueous humor was removed and replaced with isotonic deuterium oxide. At varying time intervals the decline in DTO concentration in the anterior chamber was measured. An average half-life of 2.7 minutes was found. The results of this brilliant study have been widely accepted but the rapid turnover found by these investigators may be due in part to the vascular disturbance caused by the initial paracentesis. In the series in which the paracentesis was done after intraperiotoneal injection, the values are in much closer agreement with those reported here.

## Summary

Tritiated water was used as a tracer substance to determine the steady-state rate of turnover of anterior aqueous humor in rabbits. The rate of turnover was estimated to be between 5.2 percent per minute and 15.6 percent per minute, a value which is in agreement with that previously reported in which the blood to aqueous methods were used.

*950 East 59th Street (37).*

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## Discussion

Dr. Langham (London, England): This interesting study indicates the difficulties of determining the turnover rate of molecules that move rapidly across the blood-aqueous barrier. In kinetic studies it has generally been considered adequate to assume the arterial concentration to be approximately equal to that in the capillaries of the iris and ciliary processes. However, if a compound is lost very rapidly from the circulation, a considerable concentration gradient between the arterial and venous circulations will develop in the early period of the experiment. This is seen strikingly for ascorbic acid which is almost, if not completely removed from the blood as it flows through the ciliary processes. In these conditions it would be incorrect to substitute Cp in the above transfer equation.

Another difficulty arises from the assumption inherent in the mathematical formulation that the resistance of the blood-aqueous barrier is the factor limiting the rate of accumulation in the aqueous humor of fast moving molecules, like water. This may not be so, for the observed tendency of the transfer constant to decrease with time in spite of the error introduced by using arterial rather than the mean capillary concentration in the equation is consistent with the view that the time taken for mixing limits the rate of accumulation in the aqueous humour. This interpretation is further supported by the wide scatter of the experimental results; thus the time taken for molecules to become evenly distributed throughout the fluid would vary according to anatomic as well as physiologic factors.

The results of this study are, I feel, in general agreement with those of Kinsey, Grant and Cogan (1942) when it is recalled that anesthesia was not used in the latter study; this variable is likely to modify the rate of mixing within the aqueous humour and could well account for the values in the present study being somewhat lower than those reported previously.

Dr. V. Everett Kinsey (Detroit): I was very happy to learn that Dr. Newell and his co-workers were going to repeat this work with heavy water, using tritiated water. I would like to suggest that the authors recompute the turnover rates using the more complete mathematical treatment now available which takes into account the amount of tritiated water moving into the anterior chamber by flow from the posterior chamber as well as the amount which flows out of the anterior chamber. In the absence of specific data on the concentration in the posterior chamber, I believe the assumption could be made that the concentration was equal to that in the blood without introducing appreciable error.

Grant, Cogan and I were aware of the fact that the diffusion rate of water in the aqueous humor itself was probably the limiting factor so far as the turnover rate was concerned. This was the chief reason for measuring the rate of transfer both in the blood-aqueous direction and in the aqueous to blood direction.

I believe that although introducing heavy water into the anterior chamber may have caused some dilitation of the iris vessels, the results obtained in these experiments may more realistically represent the rapid rate of turnover of water than the experiments in which the measurements were made in the opposite direction.

As I recall, the turnover was about 20 percent per minute. This point was also brought out some years ago by Dr. Friedenwald, who continually emphasized the fact that diffusion through the aqueous humor itself limited the turnover rate.

Dr. Frank W. Newell (closing): I wish to thank Dr. Langham and Dr. Kinsey for their valuable discussion of this paper, and to mention some of the inaccuracies inherent in steady-state measurements of a rapidly moving molecule, such as water.

Tritium is a toxic substance. It is probably not toxic in the amounts used in this study but certainly this factor must be considered. Additionally, it has a molecule which is larger than the ordinary water molecule, which may be a factor limiting its entry into the eye.

Anesthesia has a profound effect, and markedly modifies the results of a diffusion experiment such as this. The measurement of femoral arterial blood causes many inaccuracies, because we are not measuring the activity of the blood that is bathing the eye which is the solution in which we are interested.

All of these experiments are limited because one can obtain but three points—the zero point, a point from the right eye, and a point from the left eye. No steady-state study is accurate determining only the anterior chamber values, and we are well aware of this. At present we are determining posterior chamber values, but realize that even so we cannot obtain exact results. It is likely that the equilibrium is established adjacent to the blood vessels, or wherever the level of the blood-aqueous barrier is, and that our huge sample of aqueous humor reflects less tritiated water than is really in equilibrium at the point of exchange. Also, we have not measured the intraocular metabolism and the rapid exchange of water within the eye itself.

# STUDIES ON THE MODE OF ACTION OF PILOCARPINE ON AQUEOUS OUTFLOW*

MILTON FLOCKS, M.D., AND H. CHRISTIAN ZWENG, M.D.
*Palo Alto, California*

Recent tonographic studies[1,2] indicate that pilocarpine lowers the resistance to aqueous outflow in normal as well as in glaucomatous eyes. There is no agreement, however, on the way this is accomplished.

There are two principal schools of thought on the subject. Perhaps the most popular theory is the one which suggests that the way that pilocarpine reduces the intraocular pressure is by dilating the episcleral and scleral blood vessels thus reducing the resistance to outflow. In support of this theory, Thomassen[3] states that after pilocarpine instillation into an eye with increased intraocular pressure, the episcleral venous pressure fall precedes the drop in intraocular pressure on the average by 15 minutes. Alfano and Alfano[4] in an interesting study of glaucoma secondary to obstruction of the superior vena cava by mediastinal masses demonstrated that pilocarpine decreased the intraocular pressure prior to contraction of the pupil. They were of the opinion that the drug lowered the tension by reducing the pressure in the episcleral veins.

The second important theory as elaborated by Fortin[5] and others is that the drug acts chiefly by the pull of the ciliary muscle on the scleral spur and trabecula to "enlarge the spaces of Fontana and open the Canal of Schlemm." Fortin demonstrated a change in the histologic appearance of the trabecular meshwork of monkey eyes under the influence of eserine.

Other theories[6] hold, with very little evidence to support them, that the tension lowering effect of pilocarpine is produced by: (a) constriction of the ciliary muscle fibers which constrict the long posterior ciliary arteries like a sphincter, reducing the blood pressure in the anterior segment; (b) contraction of the iris mechanically increasing the efficiency of the drainage angle; (c) prolonged vasoconstriction causing a decrease in blood volume and a decreased production of aqueous humor.

In 1955, one of us (M. F.[7]) described the appearance of the trabecular meshwork of human eyes as seen in tangential section. At that time we were particularly impressed by the openings in the corneoscleral portion of the meshwork as demonstrated by this method of study. These openings were seen to be elliptical in shape with the long axes of the ellipses lying circumferentially, parallel to the scleral spur, and perpendicular to the longitudinal fibers of the ciliary muscle. Since these fibers inserted on the scleral spur, the arrangement was such that the openings should become rounder and therefore larger when the ciliary muscle contracts. This would, of course, tend to decrease the resistance to outflow and help to explain the tension lowering action of pilocarpine.

Although Becker[1] and Weekers et al.[2] have found that pilocarpine reduces the facility of outflow of normal human eyes, others[8] have found that pilocarpine lowers the tension only in glaucomatous eyes and has no effect on normal eyes. A tonographic study was undertaken to confirm in monkeys what Becker and Weekers had found to be true in humans; for if histologic changes in the trabecular meshwork following the use of pilocarpine were found, it would be essential to know whether the facility of aqueous outflow had also been effected by the drug.

* From the Division of Ophthalmology, Department of Surgery, Stanford University School of Medicine, San Francisco. This investigation was supported by research Grant B-873, United States Public Health Service.

We also decided to study goniophotographs of the trabecular areas of cyclotonic and cycloplegic eyes of monkeys to see if any measurable differences were discernible by this method.

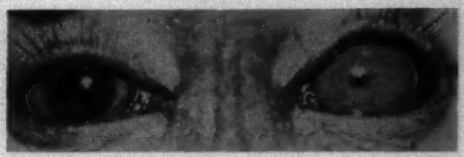

Fig. 1 (Flocks and Zweng). Prior to enucleation.

## Material and Methods

Monkey eyes were used because of the marked similarity of the trabecular meshwork of these animals to those of human eyes. In lower animals, the corneoscleral portion of the meshwork is either absent or much less prominent. Ten Rhesus monkeys and one baboon were used for the anatomic studies. In some monkeys tonography was performed on several days before the eyes were removed.

When an eye under the influence of atropine is enucleated, the pupil remains dilated. But when an eye with the pupil made miotic by frequent topical applications of pilocarpine, eserine or DFP is enucleated the pupil dilates at the time of enucleation. This is probably why so few histologic studies of the effect of miotics have been made. Van Alphen,[9] in 1951, managed to overcome this by quick-freezing the eyes in liquid nitrogen immediately prior to enucleation. With this method he obtained enucleated monkey eyes in good state of miosis. We attempted to duplicate his method using rabbits at first; but soon discovered that quick freezing the eye immediately prior to enucleation was awkward, dangerous and not entirely effective. We, therefore, devised a method of poisoning the animal with pilocarpine after the control or mydriatic eye had been removed and then enucleating the miotic eye.

One drop of two-percent pilocarpine was instilled in the left eye every five minutes six times approximately 45 minutes before tonography was to be performed.

In most of our experiments we used a cycloplegic drug in the opposite, control eye. If we did not, the amount of dilatation of the pupil in this untreated eye was very variable under anesthesia. We felt also that the use of a cycloplegic drug would increase the difference in the histologic appearance of the meshwork of the two eyes.

Usually two-percent atropine sulfate was used, but in a few cases in which we wanted to avoid prolonged cycloplegia, one-percent cyclogyl, one drop every five minutes six times was substituted for atropine. No demonstrable difference was noted between these two cycloplegic drugs as far as our studies were concerned.

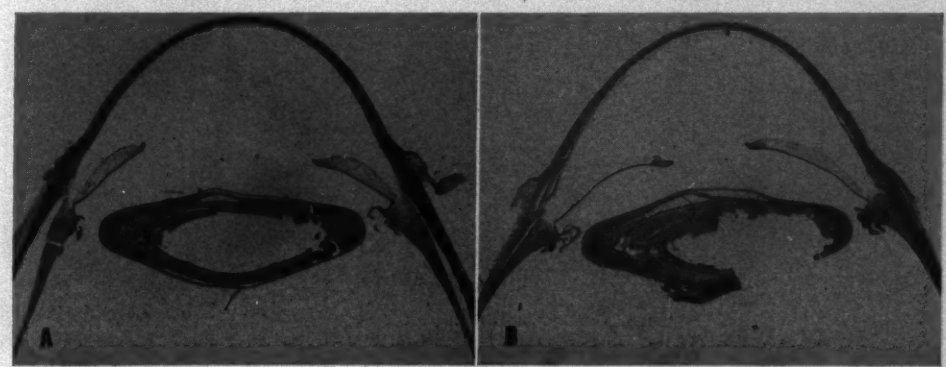


Fig. 2 (Flocks and Zweng). (A) Effect of atropine. Note large pupil and thick iris. (B) Effect of pilocarpine. Note small pupil and thin iris.

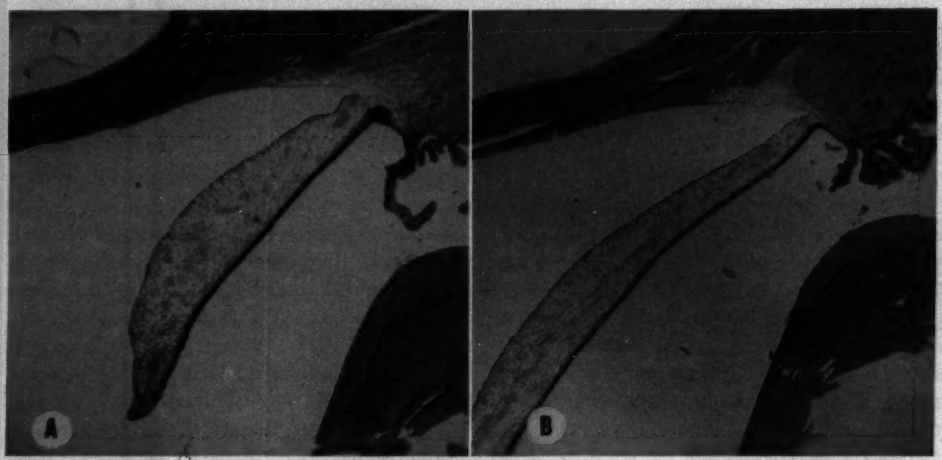


Fig. 3 (Flocks and Zweng). (A) Effect of atropine. The lamellae of the meshwork are relatively close together. (B) Effect of pilocarpine. The lamellae of the meshwork are spread. (Meridional sections.)

A marked mydriasis was quickly achieved in the right eye and a marked miosis in the left eye (fig. 1).

Interperitoneal nembutal anesthesia was used with the addition of ether by inhalation in some cases.

When satisfactory anesthesia was obtained tonography was performed. Sometimes it was done on the right eye first and in some cases on the left eye first. The lids were held apart by an assistant when tonography was being performed. In one instance the lids were removed 10 minutes prior to tonography to make sure that the pressure of the lids from the small lid apertures was not producing erroneous results. In about one fourth of the trials a conjugate deviation of the eyes which we attributed to the

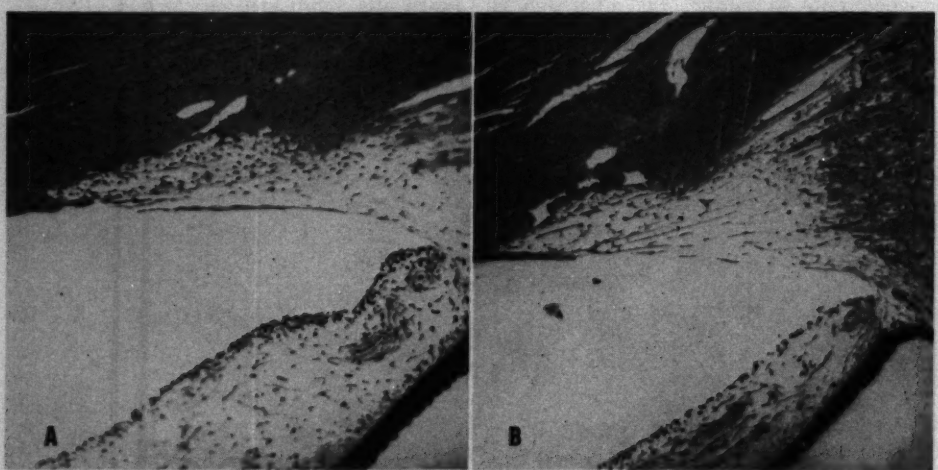


Fig. 4 (Flocks and Zweng). (A) Effect of atropine. (B) Effect of pilocarpine. The lamellae of the meshwork are spread. The scleral spur is pulled posteriorly by the ciliary muscle.

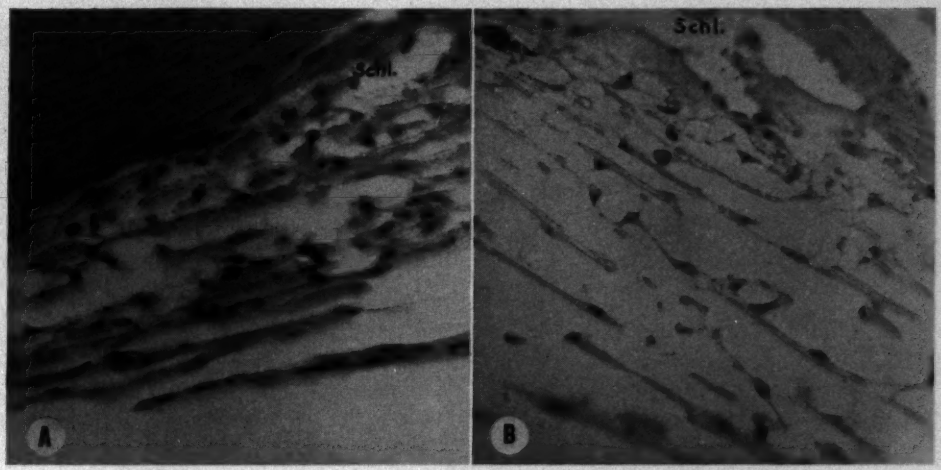


Fig. 5 (Flocks and Zweng). (A) Effect of atropine. (B) Effect of pilocarpine. Same as Figures 3 and 4. High power.

anesthetic drug caused the tracings to be unsatisfactory and these were discarded.

The right, cycloplegic eye was then enucleated and placed in formalin. An intraperitoneal injection of 10 times the lethal dose of pilocarpine was given. The effect of the toxic dose of pilocarpine usually first became apparent by urination and slowed respiration. Within 10 to 15 minutes after the injection, enucleation of the left eye was begun.

Meridional sections of the eyes were prepared in celloidin in the usual manner and stained with hematoxylin-eosin and Masson's trichrome stains. Tangential sections were prepared in the manner previously described by one of us.[7]

On other occasions prior to tonography

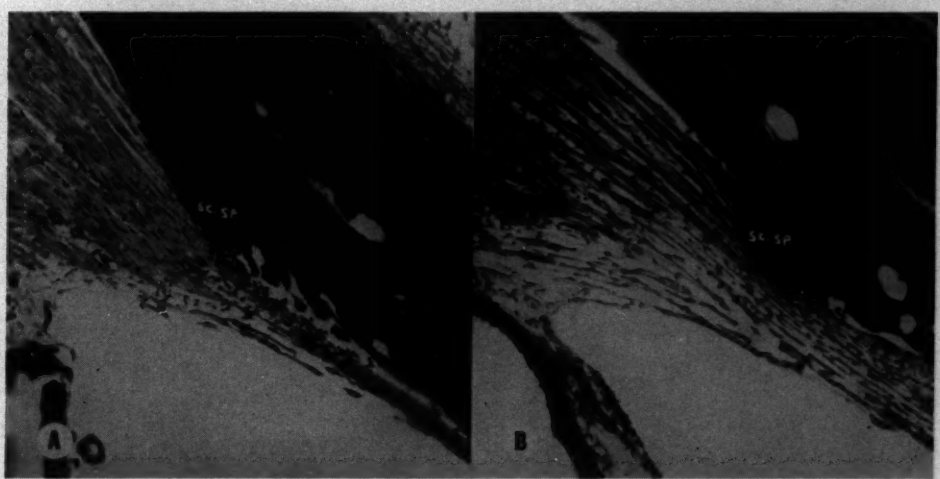


Fig. 6 (Flocks and Zweng). (A) Effect of atropine. Scleral spur is not pulled posteriorly. (B) Effect of pilocarpine. Scleral spur is pulled posteriorly.

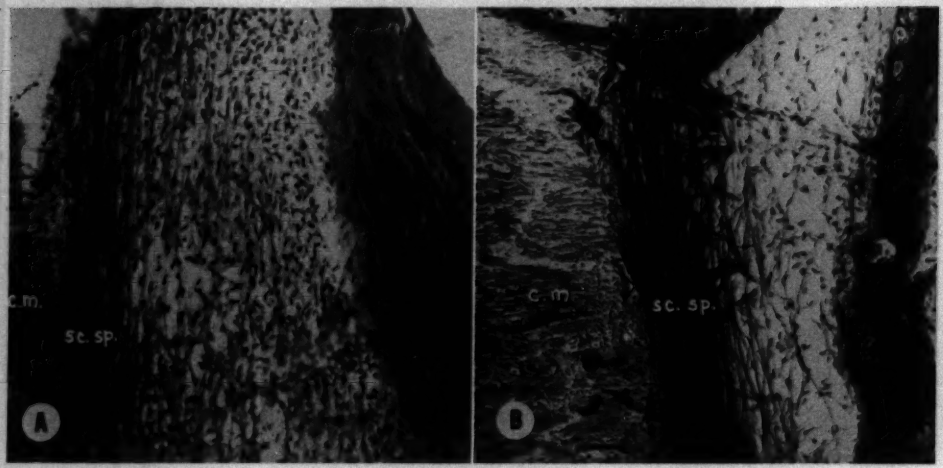


Fig. 7 (Flocks and Zweng). Effect of atropine. Note relationship of C.M. to sc. sp. Trabecular openings are narrow. (B) Effect of pilocarpine. Trabecular openings are large. (Tangential sections.)

and enucleation in some of these animals goniscopy was performed on the eyes treated with pilocarpine and atropine as described above. Gonioscopic pictures were taken. The trabecular areas were measured with a micrometer on the pictures to determine if there was a difference in size between the meshwork of the cyclotonic and cycloplegic eye.

## Results

### 1. Anatomic

The trabecular meshwork of the cyclotonic eyes looks strikingly different from the meshwork of the cycloplegic eyes. Differences are easily seen in both meridional and tangential sections.

In meridional sections the trabecular la-

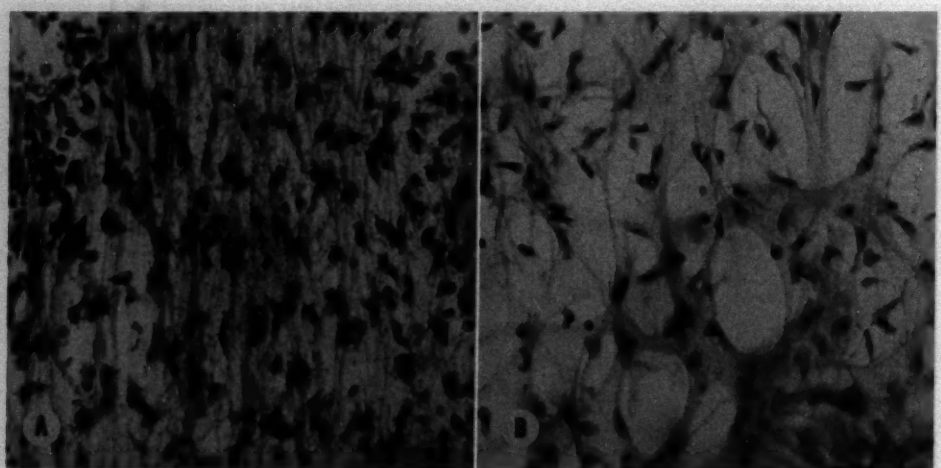


Fig. 8 (Flocks and Zweng). (A) Effect of atropine. Meshwork openings are narrow. (B) Effect of pilocarpine. Meshwork openings are large. Nuclei are farther apart. (Tangential sections, high power.)

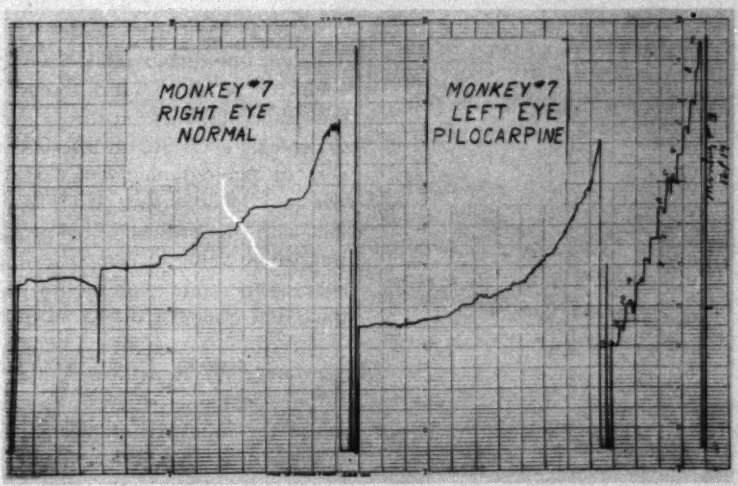


Fig. 9 (Flocks and Zweng). Aqueous outflow is greater in the cyclotonic eye than in the untreated eye.

mellae become farther apart and appear stretched under the influence of pilocarpine (figs. 3, 4, and 5). The scleral spur is pulled posteriorly by the action of the longitudinal fibers of the ciliary muscles (fig. 6).

In tangential sections the elliptical openings in the corneoscleral meshwork are rounder and larger in the eyes treated with pilocarpine (figs. 7 and 8). This produces a much more open appearance of the meshwork in these eyes.

### 2. Tonographic

Pilocarpine causes a definite increase in the facility of outflow of normal monkey eyes (table 1, figs. 9 and 10). The differ-

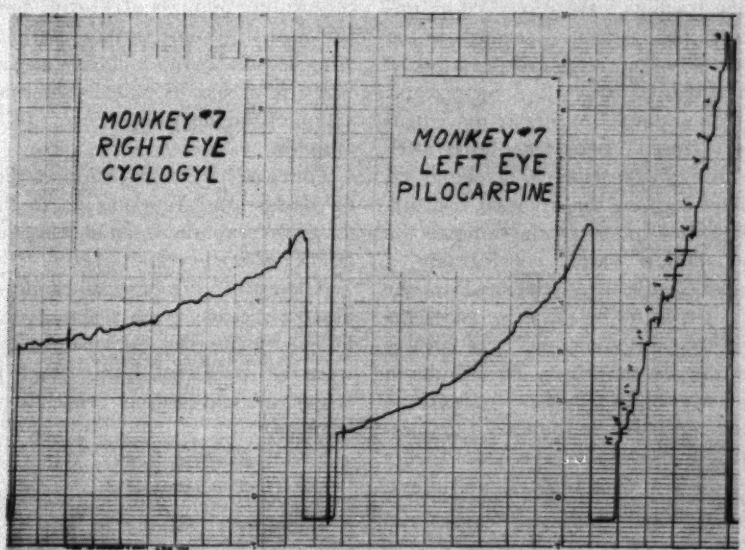


Fig. 10 (Flocks and Zweng). Aqueous outflow is greater in the cyclotonic eye than in the cycloplegic eye.

TABLE 1

APPARENT* FACILITY OF AQUEOUS OUTFLOW (C)†

| Trial | Pilocarpine | Cycloplegic Drug | No Drug |
|---|---|---|---|
| 1 | 0.73 | 0.49 | |
| 2 | 0.51 | 0.36 | |
| 3 | 0.56 | | 0.35 |
| 4 | 0.51 | 0.15 | |
| 5 | 0.36 | 0.23 | |
| 6 | 0.61 | 0.52 | |
| 7 | 0.37 | 0.29 | |
| 8 | 0.51 | | 0.32 |
| 9 | 0.61 | 0.33 | |
| 10 | 0.25 | 0.18 | |
| 11 | 1.33 | 0.25 | |
| 12 | 0.53 | 0.52 | |
| 13 | 0.47 | 0.28 | |
| 14 | 0.61 | 0.30 | |

* We realize that because of the many sources of error and the technical difficulties involved in performing tonography on monkey eyes, these figures do not express the true facility of outflow. The figures are listed merely to show the extent of the differences between the pilocarpine-treated eye and the opposite eye.

† Using 1955 Friedenwald table corrected for ΔP.

ence appeared even more marked when the tonographic tracings of the cyclotonic eye are compared with those of the cycloplegic eye of the same animal.

Because of technical difficulties about one third of the tracings were discarded as unsatisfactory. The difficulty seemed to be chiefly the state of anesthesia of the animals. The most common cause of a poor result was a sudden sustained conjugate movement of the eyes. Inhalation of ether used after the preliminary intraperitoneal nembutal did not definitely improve the situation. On different occasions in the same animal the facility of outflow varied considerably, a variation which we also attributed to the anesthesia; but in all of these instances the facility in the cyclotonic eye was always higher than in the other eye. The results in the large majority of the tracings were so definite and consistent as to make it unquestionable that the cyclotonic eye had a higher facility of outflow than the cycloplegic eye.

### 3. Gonioscopic

From our gonioscopic photographs we could not be sure of a difference in size of the trabecula between the cyclotonic and cycloplegic eyes. Slight differences in sharpness of photographic image and the small size of the structure itself convinced us that with our present technique, gonioscopy was of no value in demonstrating a difference in the trabecular meshwork of cyclotonic and cycloplegic eyes of monkeys.

## Summary and Conclusions

Our anatomic studies of monkey eyes under the influence of pilocarpine indicate that the drug acts to decrease the resistance to aqueous outflow by its action on the ciliary muscle which produces striking changes in the trabecular meshwork.

In serial tangential sections of the corneoscleral meshwork, the normally elliptical openings of monkey eyes under the influence of pilocarpine are seen to be rounder and therefore larger than those openings in the fellow cycloplegic eye of the same monkeys.

Meridional sections of these same eyes confirm the findings of Fortin. The trabecular lamellae become farther apart and appear stretched under the influence of pilocarpine. The scleral spur is pulled posteriorly by the action of the longitudinal fibers of the ciliary muscles.

Tonographic studies support the findings of Becker and Weekers et al. that miotic drugs decrease the resistance to aqueous outflow of normal eyes.

Attempts to demonstrate changes by gonioscopic means in the trabecular meshwork in eyes under the influence of pilocarpine were not successful. In our hands the gonioscopic method is too crude to show the changes.

*850 Middlefield Road.*
*300 Homer Avenue.*

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## Discussion

Dr. Bernard Becker (Saint Louis): It is a very pleasant task to discuss this paper. It is always pleasant to discuss a paper that confirms something one has written several years ago, at least in part.

As Dr. Zweng has pointed out so well, it has been known for many years that pilocarpine reduces intraocular pressure in glaucomatous eyes, and it also produces a small fall in intraocular pressure in normal human eyes. That dates back to the work of Golowin in 1895 and Schiøtz in 1905. The mechanism of this decrease in intraocular pressure has been a subject of considerable speculation.

The present paper presents histologic and tonographic evidence that pilocarpine decreases the resistance of aqueous outflow through the trabecula. The histologic pictures are impressive and the steepening of the tonographic tracings in monkey eyes is even greater than that seen in patients. Notes of caution must be expressed, however, as to the underlying effects of general anesthesia and as to the interpretation of tonography values in monkey eyes. Furthermore, one must decide whether the steeper tracing in the pilocarpine-treated eye results from an increase in outflow facility, an increase in the coefficient of scleral rigidity or both. In addition, one must rule out the possibility of an apparent steepening of the tracing because the intraocular pressure is falling to a new steady-state.

The authors are to be congratulated on their accomplishments to date. It is hoped that the verification of their conclusions will be forthcoming. This will require perfusion studies, scleral rigidity measurements, and determinations of the chemical composition of the aqueous humor.

Concerning the study of scleral rigidity, I might add as an historical note that the first description of a fall in intraocular pressure in normal eyes following pilocarpine administration was done by Golowin in 1895, and at that time the only tonometer available was the applanation one of Maklakoff. The fall in intraocular pressure was obtained as measured by this instrument which is reasonably independent of scleral rigidity. I think this should be confirmed, however, with the more modern methods of measuring scleral rigidity.

Dr. Frank Vesey (Toledo, Ohio): The trabecular structure may be concerned with other functions beside imbibing the aqueous and perhaps introducing it into Schlemm's canal, which is a very small structure as compared to the tremendously large vascular bed of the uvea; but the trabecular, spongy tissue itself may well be concerned with the nutrition of the cornea.

The cornea must be nourished from somewhere. The superficial vessels coming from the episcleral tissue and from the conjunctiva would not be enough to nourish that very important structure of the transparent cornea. It might be that the trabecular, spongy tissue takes up the nourishing elements from the aqueous and conveys it to the deeper layers of the cornea.

In my experiments, in blocking the anterior chamber angle with polyethylene tubing by purely mechanical means, a very interesting phenomenon occurred. After two or three days a crimson ring appeared around the limbus, about two mm. wide, leaving the center of the cornea transparent. At first it was so dense that I thought I was dealing with a hemorrhage in the anterior chamber, but by finer methods of examination and larger magnification it turned out to be a deep vascularization of the cornea. I speculated that this ring was a substitute for the lost nourishment of the trabecular tissue momentarily.

Dr. Howard Stone: I have one question that I would like to ask Dr. Becker. Could the mechanism demonstrated here, or a similar one, be used to explain the fact (as some investigators have recently found) that the facility of outflow obtained by tonography increases with accommodation?

Dr. H. Christian Zweng (closing): I should like to thank Dr. Becker, Dr. Vesey and Dr. Stone for their comments on our efforts. We are certainly wholeheartedly in agreement with Dr. Becker when he says that tonographic evaluation is difficult in the monkey. We examined our tonograms with a very critical eye and, as we said, we discarded a number of them because we felt they did present

technical difficulties.

The tonograms did vary in the same animal and in the same eye from one week to another. However, as we pointed out in the body of the paper, never did the outflow curve in the pilocarpinized eye fail to be steeper than in the opposite eye.

As to the remarks concerning scleral rigidity and the studies on perfusion and chemistry, changes which perhaps are induced by the medication, we will take them back to San Francisco with us.

It is always a great pleasure to have a paper discussed by Dr. Becker, because in one minute he is able to suggest enough activity to keep us busy for approximately one decade.

As to Dr. Stone's question concerning whether or not this perhaps gives an explanation for the increase of facility of outflow and accommodation, we feel that is a real possibility; and, as a matter of fact, we are engaged in studying that very point now.

Concerning Dr. Vesey's remarks, I believe it is beyond our scope here to review the evidence which points to the trabeculum as at least an important source of aqueous absorption. We can only say in reference to our own paper that we feel we have demonstrated a change in outflow facility in the same eyes in which we have demonstrated histologic changes in the trabecular spaces. I suppose it is always possible that the two are co-incidental. We can only leave it at that.

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# ELABORATION OF BICARBONATE ION IN INTRAOCULAR FLUIDS*

## IV. Dynamic effects of acetazolamide (Diamox)

Harry Green, Ph.D., Mayer J. Mann, A.B., and Henry S. Kroman, M.S.
*Philadelphia, Pennsylvania*

In view of the role that carbonic anhydrase is believed to play in the $CO_2$ transport by the red cell, in the reabsorption of bicarbonate ion by the kidney tubule and in the secretion of an alkaline fluid by the pancreas, it was reasoned analogously by most investigators and clinicians that ophthalmologically speaking the primary target of acetazolamide, a potent carbonic anhydrase inhibitor, was the enzyme present in the ciliary processes.

The demonstration by Green and coworkers[1,2] that the intravenous administration of acetazolamide (Diamox) to rabbits completely inhibited the carbonic anhydrase activity of the iris and ciliary body within 30 minutes lent support to the possibility that the drug exerts its hypotensive action on glaucomatous eyes by depressing the rate of elaboration or active transport of bicarbonate ion into the posterior chamber of the eye.

An objection to the acceptance of this concept of the mechanism of action of the drug was presented[3] when it was found that, although the carbonic anhydrase activity of the iris and ciliary body of the rabbit eye was completely inhibited following the intravenous and subconjunctival injection of Diamox, the bicarbonate ion concentration in the anterior aqueous humor was substantially unchanged (30 to 60 minutes after intravenous injection and up to three hours following subconjunctival injection).

At the same time there was no significant lowering of the arterial blood plasma bicarbonate concentration. Subsequently, it was shown that the bicarbonate ion concentration in the posterior aqueous humor was also unaffected by the subconjunctival injection of the drug.[4]

As far as the intraocular pressure was concerned some rabbits showed no significant lowering[2] while others were reported to respond to the intravenous administration of the drug with a marked decrease.[5] (For an explanation of the difference between "responsive" and "nonresponsive" rabbits, see Becker and Constant.[6]) On the other hand,

* From the Research Department, Wills Eye Hospital and the Department of Biochemistry, Graduate School of Medicine, University of Pennsylvania. This investigation was supported in part by a grant-in-aid from the National Council to Combat Blindness.

it is generally agreed that Diamox exerts no appreciable effect upon the intraocular pressure of normal rabbit eyes when given subconjunctivally or by any other local route.[4]

At the time that Green and coworkers were conducting their experiments on the effect of Diamox upon the bicarbonate-ion concentration in the anterior chamber of the rabbit eye, and, indeed, up to the time of the publication of their results,[3] the kinetics of penetration of test substances into the posterior chamber and the rate of turnover of ions in that chamber were not known. On the basis of the information then available concerning the steady-state distribution of bicarbonate ion between the aqueous humor in the posterior and anterior chambers it was reasonable to assume that "any interference with the active transfer of bicarbonate ion into the posterior chamber would be reflected in a lowering of the concentration of the ion in the anterior chamber." Furthermore, since, as Becker[5] had shown the intraocular pressure-lowering effect of Diamox upon "responsive" rabbits could be elicited within the first hour, it was reasonable to expect any change in the bicarbonate-ion concentration in the anterior chamber to occur during this interval of time.

The first experimental evidence that the kinetics of penetration of test substances into the posterior chamber are different from those into the anterior chamber was published by Kinsey, Palm and Cavanaugh[7] at the same time that the results of Green and co-workers, already referred to, appeared. Using thiocyanate and radioactive $Na^{24}$ ions as test substances, Kinsey and co-workers demonstrated that during the first 20 or 30 minutes following intraperitoneal injection the initial rate of penetration into the posterior chamber was greater than into the anterior chamber.

On this basis it is reasonable that an intravenous administration of Diamox could have a significant lowering effect on the bicarbonate-ion concentration in the aqueous humor of the posterior chamber within a short period of time without appreciably affecting the concentration in the anterior chamber. Furthermore, it is reasonable to assume that a longer period of time is required for any significant drop in the bicarbonate-ion concentration in the anterior chamber to occur, as suggested by Becker[8] (see later).

In the study of the mechanism of action of Diamox, Becker[8] observed that a single intravenous administration of 100 mg./kg. caused a marked drop in the bicarbonate-ion concentration of the posterior chamber fluid in 25 minutes. During the same interval of time, however, there was no significant change in the bicarbonate-ion concentration of the anterior chamber fluid, or of the blood plasma, a verification of the results of Green and co-workers. Kinsey, in a private communication to one of us (H. G.), similarly found no significant drop in the bicarbonate-ion concentration of the anterior chamber fluid of the rabbit eye 60 minutes after a single intravenous administration of Diamox; the concentration in the posterior chamber fluid, however, fell significantly.

As already mentioned, the short-term experiments (approximately 25 minutes) of Becker were conducted with a single administration of Diamox. For long-term experiments, however, Becker administered the drug intravenously to rabbits in doses of 100 mg./kg., followed by 25 mg./kg. every 30 minutes for six hours, in order to prolong the inhibition of the carbonic anhydrase activity. Under the latter conditions the bicarbonate-ion concentration in the aqueous humor of both the anterior and posterior chambers fell significantly at the end of the six-hour period while that of the blood plasma was unaffected. In view of these results Becker concluded that the failure of Green and co-workers to find significant changes in the anterior chamber bicarbonate 60 minutes after Diamox administration was due to the fact that the experimental time period was too short. Furthermore, he took issue with the conclusions reached by Green

et al. that carbonic anhydrase activity of the anterior uvea may not be involved in the elaboration or maintenance of bicarbonate-ion concentration in the aqueous humor of rabbit eyes, but stated that, "there is every indication that aqueous bicarbonate concentrations as well as the rate of secretion of aqueous humor are dependent upon carbonic anhydrase activity."

The conclusions by Becker, it is to be noted, are based upon the administration of repeated doses of Diamox over a period of six hours. Maren and co-workers,[9] in a detailed and fairly exhaustive study of the effects of the drug, have shown that the continued administration of high doses of Diamox to dogs and rats produced a state of profound and sustained metabolic acidosis. It is quite probable, therefore, that under the conditions of continued administration of high doses of Diamox to rabbits employed by Becker a similar state of acidosis was produced. If this is so, then the primary effect of repeated doses of the drug upon the bicarbonate-ion concentration in the aqueous humor may not be inhibition of the carbonic anhydrase of the ciliary body, as Becker has assumed, but may be fundamentally associated with a disruption of the bicarbonate-ion balance between the blood, the intraocular fluids and the extracellular fluid of the tissues, associated with a state of metabolic acidosis.

Accordingly, the present investigation was undertaken to study the effect of an intravenous injection of a single dose of Diamox upon the pH and bicarbonate-ion concentrations of the arterial blood plasma and of the aqueous humors in the anterior and posterior chambers of the rabbit eye over varynig periods of time. At the same time it was expected that a detailed kinetic picture of the turnover rates of the bicarbonate ion in the respective fluids could be achieved.

While the present investigation was in progress a report appeared by Langham and Lee[10] in which they observed that the intravenous administration of a single dose of Diamox caused an immediate but temporary reduction in the intraocular pressure, in the rate of formation of aqueous humor and in the excess of bicarbonate in the anterior aqueous humor of the rabbit eye. It was suggested that these phenomena were associated with the action of the drug in inducing a rapid fall in the pH of the aqueous humor, that is, within 15 minutes. In conditions of steady-state, however, as two to three hours after repeated acetazolamide treatment, the distribution ratios returned to normal. However, it must be noted that only the aqueous humor of the anterior chamber was analyzed!

## Methods and Procedures

Diamox* (100 mg. of the sodium salt/ml.) was given intravenously in a dose of 30 mg./kg. body weight to adult colored rabbits weighing between 2.0 and 3.0 kg. Bicarbonate analyses were determined in a Warburg apparatus at 37°C. in an atmosphere of 5.0-percent $CO_2$ as previously described.[3] Single side-arm flasks of 7.0 or 15 ml. capacity were used depending upon the size of the sample analyzed. Posterior chamber samples of aqueous humor were usually between 20 to 30 µl.† while samples of the anterior chamber fluid and blood plasma were 0.1 to 0.2 ml.

The aqueous humor was removed from a proptosed eye previously treated with one drop of 0.5-percent pontocaine hydrochloride. Prior to paracentesis the eye was flushed with isotonic saline and blotted dry. The posterior chamber aqueous humor was removed by means of a capillary pipette with a sealed-in platinum 26-gauge needle as previously described.[4] Immediately, following

* Diamox was generously supplied by the Lederle Laboratories Division, American Cyanamid Company, through the kindness of Dr. J. D. Gallagher and Dr. C. H. Demos.

† It was found necessary to dilute all posterior-chamber samples to at least 0.1 ml. with $H_2O$ in the Warburg flask in order to avoid unstable pressure readings arising from insufficient liquid volume.

paracentesis of the posterior chamber the aqueous humor of the anterior chamber was removed as previously described.[2]

Blood plasma was prepared from whole blood withdrawn from the central ear artery into a heparinized syringe as previously indicated.[11]

The pH of whole blood and of the aqueous humor of the anterior chamber was measured by a Cambridge Research Model pH meter, manufactured by the Cambridge Instrument Co., Inc., New York, using a micro condenser type glass electrode SK-5697. By this means the samples of the fluid could be drawn directly from the animal into the glass cell under essentially anaerobic conditions and the pH measured within one minute. The pH measurements were steady for at least five minutes and no special precautions of preparing the glass cell other than washing with glass-distilled water was necessary. For blood the cell was heparinized before use. By proper assembly of the instrument as little as 0.2 ml. of fluid was found suitable for a measurement with an accuracy of 0.01 pH units. The pH values observed at room temperature were corrected to 37°C. using the correction factors of Langham and Lee[10] (whole arterial blood, 0.0147 pH units/°C. and aqueous humor, 0.0064 pH units/°C.).

## Experimental

In all cases one eye (O.S.) was used as a control for the zero minute value while the aqueous humors of the contralateral eye (O.D.) were analyzed at the indicated time period. Blood was analyzed also at zero minutes and at the experimental time period. The pH studies were done on a separate series of animals. Each animal was used only once for an experiment.

## Results

*Effect of prior posterior chamber tap upon the bicarbonate concentration in the aqueous humor of the same eye* (table 1). The results show that the procedure of removing the aqueous humor from the posterior chamber immediately prior to removing the aqueous humor from the anterior chamber had no apparent effect upon the bicarbonate concentration in the latter.

TABLE 1

EFFECT OF PRIOR POSTERIOR CHAMBER TAP UPON THE BICARBONATE CONCENTRATION IN THE AQUEOUS HUMOR OF THE ANTERIOR CHAMBER

Left eye (O.S.), anterior chamber fluid removed after paracentesis of posterior chamber; right eye (O.D.), anterior chamber fluid removed without prior paracentesis of posterior chamber.

| | O.S. mM/l | O.D. mM/l | Dif. |
|---|---|---|---|
| | 35.9 | 35.1 | −0.8 |
| | 30.4 | 31.2 | +0.8 |
| | 31.5 | 30.7 | −0.8 |
| | 32.7 | 31.6 | −0.9 |
| | 31.0 | 29.2 | −1.8 |
| | 34.9 | 36.4 | +1.5 |
| | 35.3 | 34.1 | −1.2 |
| | 28.2 | 28.0 | −0.2 |
| | 27.7 | 28.6 | +0.9 |
| Average | 32.0 | 31.7 | −0.28 |
| S.E. | 0.33 | 0.30 | 0.15 |

Absolute difference between two eyes, 1.0

*Effect of Diamox (30 mg./kg., I.V.) upon the bicarbonate concentration* (table 2 and fig. 1). A summary of the numerical results are given in Table 2, in which the average values of N samples of fluid removed before ($t_o$) and after Diamox administration (time t) are in millimoles of bicarbonate per liter of solution. The column % Dif. has been calculated as the change in the bicarbonate concentration from the $t_o$ value. The horizontal row S.E. gives the standard errors of the values in the row immediately above. Because the average $t_o$ values vary somewhat in the different experiments the graphical representation of the data is in the form of percent change in bicarbonate concentration with time of duration of the Diamox treatment.

### A. *Aqueous humor of the posterior chamber*

The bicarbonate concentration in the posterior chamber fell the fastest, with a de-

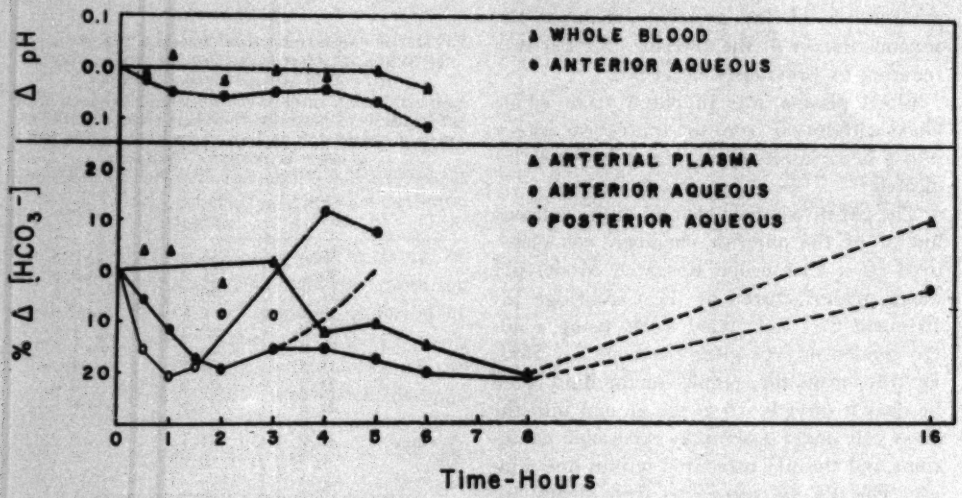


Fig. 1 (Green, Mann and Kroman). Effect of Diamox (30 mg./kg.).

crease of 15 percent in 30 minutes, and a maximum drop of 21 percent in 60 minutes. The return to normal was almost as rapid, the original concentration having been restored in about three hours. Indeed, the tendency was for the bicarbonate concentration to continue to rise past the original concentration by about 12 percent during the fourth hour and then decrease to the baseline.

### B. *Aqueous humor of the anterior chamber*

In previous investigations it had been reported that no significant lowering of the bicarbonate concentration in the anterior chamber fluid occurred in 30 to 60 minutes following the administration of the drug. The present results indicate, however, that although during the early time period the fall in bicarbonate concentration was slight, nevertheless this trend was continuous, so that after 90 and 120 minutes the bicarbonate concentration in the aqueous humor of the anterior chamber had fallen 17 percent and 19 percent, respectively. During the third hour the bicarbonate concentration rose from a 19-percent decrease to a 15-percent decrease, after which the concentration slowly fell again to the level of a 20-percent decrease and remained at this low level at least until the end of the eighth hour. Sometime during the next eight hours the bicarbonate concentration in the anterior chamber fluid returned to the original normal level (indicated by the broken line).

### C. *Arterial blood plasma*

The bicarbonate concentration of the arterial blood plasma was essentially unchanged during the first three hours of Diamox treatment. Then the concentration fell slowly during the next three hours, reaching a decrease of 22 percent at the end of the eighth hour. During the next eight hours the bicarbonate concentration returned to the original normal level (indicated by the broken line).

*Effect of Diamox (30 mg./kg., I.V.) upon the pH.* A summary of the numerical results is given in Table 3, in which the averages and standard errors of the mean (S.E.) of N samples of fluid removed before ($t_o$) and after Diamox administration (time t) are recorded. The differences (Column "Dif.")

TABLE 2

SUMMARY OF RESULTS: EFFECT OF ACETAZOLAMIDE UPON THE BICARBONATE CONCENTRATION

All values given are averages of N experiments.

| Time (t) (hr) | Anterior Chamber | | | | Posterior Chamber | | | | Arterial Plasma | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $t_0$ mM/l | t mM/l | % dif. | N | $t_0$ mM/l | t mM/l | % dif. | N | $t_0$ mM/l | t mM/l | % dif. | N |
| 0.5 | 30.2 | 28.1 | − 5.8 | 24 | 34.5 | 29.0 | −15.4 | 16 | 21.3 | 21.9 | + 3.7 | 24 |
| S.E. | 0.84 | 0.84 | 1.70 | | 1.3 | 0.88 | 2.4 | | 9.92 | 0.63 | 1.70 | |
| 1.0 | 31.5 | 27.8 | −11.5 | 5 | 39.0 | 30.8 | −20.9 | 6 | 21.0 | 21.0 | + 3.5 | 4 |
| S.E. | 1.1 | 0.75 | 2.76 | | 1.4 | 0.58 | 1.63 | | — | — | — | |
| 1.5 | 31.9 | 26.4 | −17.3 | 9 | 39.4 | 32.4 | −18.7 | 10 | — | — | — | |
| S.E. | 0.72 | 0.72 | 2.10 | | 0.62 | 1.2 | 2.00 | | — | — | — | |
| 2.0 | 35.7 | 28.8 | −19.4 | 9 | 39.4 | 36.0 | − 8.6 | 9 | 22.6 | 21.4 | − 2.5 | 9 |
| S.E. | 0.83 | 1.0 | 1.70 | | 0.67 | 0.73 | 1.25 | | 1.3 | 1.4 | 5.60 | |
| 3.0 | 31.0 | 26.3 | −15.2 | 6 | 35.5 | 32.3 | − 9.0 | 6 | 17.5 | 18.3 | + 1.7 | 12 |
| S.E. | 0.74 | 0.61 | 1.30 | | 9.98 | 0.82 | 1.06 | | 1.0 | 0.40 | 4.1 | |
| 4.0 | 30.1 | 25.5 | −15.2 | 6 | 37.2 | 41.5 | +12.0 | 6 | 22.1 | 18.9 | −12.1 | 9 |
| S.E. | 0.74 | 0.98 | 1.7 | | 1.38 | 1.63 | 4.3 | | 1.4 | 0.73 | 3.0 | |
| 5.0 | 33.3 | 27.4 | −17.3 | 18 | 36.0 | 39.3 | + 7.6 | 10 | 21.2 | 18.5 | −10.6 | 17 |
| S.E. | 0.64 | 0.80 | 2.15 | | 0.70 | 1.65 | 4.3 | | 1.07 | 0.75 | 2.50 | |
| 6.0 | 31.3 | 24.9 | −20.2 | 6 | | | | | 20.1 | 17.1 | −14.8 | 15 |
| S.E. | 1.08 | 1.21 | 3.17 | | | | | | 0.62 | 0.71 | 2.70 | |
| 8.0 | 35.4 | 27.5 | −21.8 | 6 | | | | | 26.7 | 20.6 | −21.7 | 6 |
| S.E. | 1.38 | 0.65 | 2.98 | | | | | | 1.30 | 0.69 | 4.10 | |
| 16.0 | 32.3 | 31.0 | − 3.7 | 6 | | | | | 20.2 | 22.2 | +10.0 | 6 |
| S.E. | 1.26 | 1.22 | 2.70 | | | | | | 1.47 | 1.75 | 2.50 | |

between the values of $t_0$ and t are plotted against time in Figure. 1.

### A. *Aqueous humor of the anterior chamber*

The pH of the aqueous humor fell slightly (0.053 units) during the first two hours, then showed a slight rise during the third hour, after which time it again continued to fall during the next three hours.

### B. *Whole arterial blood*

The pH of blood remained substantially unchanged during the first five hours and showed a slight drop of 0.038 units during the sixth hour. In a separate series of rabbits in which the pH was determined on the same animal at varying periods of time following the injection of Diamox, substantially the same results were obtained.

*Comparison of pH of aqueous humor of anterior chamber of left eye (O.S.) with right eye (O.D.) of rabbit.* The results in Table 4 show that there is no significant difference, on the average, between the pH

TABLE 3

SUMMARY OF RESULTS: EFFECT OF DIAMOX UPON pH

All values given are averages of N experiments corrected to 37°C.

| Time (t) Hours | Anterior Chamber | | | | Arterial Plasma | | | |
|---|---|---|---|---|---|---|---|---|
| | $t_0$ | t | Dif. | N | $t_0$ | t | Dif. | N |
| 0.5 | 7.598 | 7.575 | −0.023 | 6 | 7.391 | 7.382 | −0.009 | 7 |
| S.E. | 0.015 | 0.032 | 0.013 | | 0.016 | 0.020 | 0.010 | |
| 1.0 | 7.576 | 7.532 | −0.044 | 14 | 7.404 | 7.423 | +0.019 | 14 |
| S.E. | 0.016 | 0.016 | 0.017 | | 0.015 | 0.013 | 0.011 | |
| 2.0 | 7.564 | 7.511 | −0.053 | 12 | 7.411 | 7.387 | −0.024 | 12 |
| S.E. | 0.021 | 0.020 | 0.012 | | 0.018 | 0.022 | 0.016 | |
| 3.0 | 7.570 | 7.525 | −0.045 | 12 | 7.397 | 7.392 | −0.005 | 12 |
| S.E. | 0.018 | 0.018 | 0.018 | | 0.016 | 0.018 | 0.011 | |
| 4.0 | 7.589 | 7.549 | −0.040 | 6 | 7.403 | 7.388 | −0.015 | 9 |
| S.E. | 0.018 | 0.025 | 0.034 | | 0.017 | 0.054 | 0.062 | |
| 5.0 | 7.574 | 7.510 | −0.064 | 8 | 7.393 | 7.389 | −0.004 | 9 |
| S.E. | 0.023 | 0.016 | 0.017 | | 0.024 | 0.013 | 0.023 | |
| 6.0 | 7.602 | 7.485 | −0.117 | 6 | 7.360 | 7.322 | −0.038 | 6 |
| S.E. | 0.015 | 0.019 | 0.032 | | 0.014 | 0.027 | 0.025 | |

TABLE 4

COMPARISON OF pH OF AQUEOUS HUMOR OF ANTERIOR CHAMBER OF LEFT EYE (O.S.) WITH RIGHT EYE (O.D.) OF RABBIT

All values corrected to 37°C.

| | O.S. | O.D. | Dif. |
|---|---|---|---|
| | 7.569 | 7.636 | +0.067 |
| | 7.606 | 7.530 | −0.076 |
| | 7.509 | 7.551 | +0.042 |
| | 7.629 | 7.562 | −0.067 |
| | 7.618 | 7.579 | −0.038 |
| | 7.624 | 7.536 | −0.088 |
| | 7.586 | 7.582 | −0.004 |
| | 7.549 | 7.569 | +0.020 |
| | 7.511 | 7.518 | +0.007 |
| Average | 7.578 | 7.563 | −0.015 |
| S.E. | 0.014 | 0.007 | 0.010 |
| Absolute difference between two eyes, | | | 0.045 |
| S.E. | | | 0.015 |

of the left eyes and the right eyes of a series of rabbits. However, there is an average absolute difference of 0.045 pH units with a standard error of 0.010 between the two eyes of a series of rabbits.

## DISCUSSION

The results of this investigation demonstrate that the time course of action of a single dose of Diamox (30 mg./kg.) upon the bicarbonate concentration in the aqueous humor of the anterior and posterior chambers and in the arterial blood plasma is different. During the first three hours following the intravenous administration of the drug the bicarbonate concentration of the plasma remained substantially unchanged, the bicarbonate concentration in the aqueous humor of the posterior chamber fell about 20 percent and returned to its original normal value, and the bicarbonate concentration in the aqueous humor of the anterior chamber also fell about 20 percent, but more slowly than that in the posterior chamber, and already started to return to its original normal level.

During the next interval of time the tendency of the bicarbonate concentration in the aqueous humor of the anterior chamber to recover from its 20-percent decrease, presumably along the broken line after three hours (fig. 1), was arrested; instead the bicarbonate concentration temporarily remained at the reduced level during the fourth hour and then again slowly fell to the level of a 20-percent reduction during the sixth hour, where it remained at least for an additional two hours.

In the meantime the bicarbonate concentration of the aqueous humor of the posterior chamber, having returned to its original normal level in three hours, exceeded the original concentration by about 12 percent during the fourth hour, but then slowly dropped to the original level. The bicarbonate concentration in the arterial blood plasma, on the other hand, after not undergoing any appreciable change during the first three hours, fell about 15 percent during the next three hours and remained at this low level for at least until the end of the eighth hour.

Comparison of the turnover rates or the percentage changes of the bicarbonate concentration in the three fluids with time, as pictured in Figure 1, reveals many points of interest: The decrease of the bicarbonate concentration in the posterior chamber (20 percent during the first hour) and in the anterior chamber (20 percent during the first two hours) occurred while the bicarbonate concentration in the arterial blood plasma remained virtually unchanged. This independence of the bicarbonate concentration in the aqueous humors from the level in the blood plasma might suggest that the action of the drug upon the eye is not secondary to any renal or systemic effect. However, it is perhaps equally conceivable that the decrease in bicarbonate concentration in the aqueous humors could be a response to the loss of the bicarbonate through the kidney into the urine, because of the following considerations:

Maren[9] has shown that a single intravenous dose of Diamox to dogs resulted in a continuous excretion of bicarbonate ion in the urine, the maximum loss occurring

at the end of six hours. During the same time period both the bicarbonate concentration and the pH of the blood plasma fell. This sequence of events led to the condition referred to as metabolic acidosis which, as Maren has pointed out, is due to an excess of acid over base in the whole body, as determined by urinary balances. In other words, acidosis is a net gain of $H^+$ or a net loss of bicarbonate ion, the latter being referred to as "base deficit" or "base depletion," the measure of which is a measure of the acidosis.

Thus, while Diamox inhibits the renal mechanism for the reabsorption of bicarbonate ion into the blood, causing a loss of the ion in the urine, there occurs an associated withdrawal of bicarbonate ion from the extracellular body fluid, and also probably from the intracellular fluid, into the blood plasma. Depending upon the relative magnitude and rates of these two related processes of bicarbonate-ion movement at any one time, the plasma bicarbonate concentration could actually remain normal, at least during the early time periods. Indeed, this sequence of events may well explain the drop in bicarbonate concentration in the aqueous humors one and two hours after administration of the drug, while the bicarbonate concentration in the plasma remained unchanged.

Despite the concordance of the foregoing theoretical considerations with the experimental observations during the initial periods, the subsequent rise of the bicarbonate concentration in the posterior chamber to a normal level and somewhat higher after three and four hours, in the face of an actual decrease of the bicarbonate concentration in the plasma, argues very strongly in favor of a direct action of Diamox upon the mechanisms for the elaboration of bicarbonate ion into the posterior chamber.

This naturally brings into sharper focus the question whether carbonic anhydrase activity in the ciliary processes is involved in the elaboration and maintenance of the bicarbonate concentration in the posterior chamber, and, if so, to what extent. To answer this it is first of all necessary to recall that Green and co-workers[1, 2] found that a single intravenous injection of 30 mg. of Diamox/kg. body weight of rabbit resulted in a complete inhibition of the carbonic anhydrase activity of the anterior uvea in 30 and 60 minutes. Following this period of time the enzyme activity slowly tended to return to normal.

The observed behavior of the bicarbonate concentration in the posterior chamber in Figure 1 is certainly compatible with the effect of the drug upon the enzyme in the ciliary processes in that the greatest fall occurred at 60 minutes and then the concentration returned to normal. The fact that the return of the bicarbonate concentration in the posterior chamber was considerably faster than the restoration of the total enzymatic activity does not weaken this intimate relationship, since it is very probable that, similar to the situation presumably existing in the red cell and kidney tubule, only a small percentage of the total enzyme activity is necessary for the total action. However, it is important to observe that the normal excess of bicarbonate in the posterior chamber fluid over that in the arterial blood plasma is about 70 percent (37 mM/l compared with 22 mM/l), while the maximum fall in the bicarbonate concentration in the posterior chamber fluid was only 20 percent, while that in the blood plasma remained unchanged. This suggests that the maximum contribution of carbonic anhydrase activity to the elaboration of the bicarbonate ion in the posterior chamber is only 20 percent of the total and about 50 percent of the excess over the blood, the same order of magnitude as the decrease in aqueous secretion.[8]

The kinetic studies of Kinsey, Palm and Cavanaugh established that the initial rates of accumulation and the turnover rates of $CNS^-$ and $Na^{+24}$ in the aqueous humor of the posterior chamber exceeded those in the aqueous humor of the anterior chamber. This finding is consistent with the concept

of a "through-and-through circulation" of the aqueous humor from the posterior chamber forward and arises from the fact that the volume of the posterior chamber fluid of the rabbit eye is about one fifth that of the anterior chamber fluid.[7]

This situation together with the postulate of the secretion-diffusion theory that the bicarbonate ion is actively transferred into the posterior chamber, and then flows into the anterior chamber would suggest that any changes in the bicarbonate ion in the posterior chamber would be reflected in roughly similar changes in the anterior chamber, but more slowly. Indeed, the initial effects of Diamox are consistent with this expectation, in that the fall in bicarbonate concentration in the anterior chamber lagged behind that in the posterior chamber. Furthermore, the magnitude of the greatest decrease (20 percent) was the same in both chamber fluids.

From a logical continuation of this sequence of events one would expect that the recovery of bicarbonate concentration in the anterior chamber should follow that in the posterior chamber. In accordance with this reasoning the rise in the curve (fig. 1) for the anterior aqueous humor during the third hour is of particular significance and is suggestive of the initial tendency of the bicarbonate concentration in the anterior chamber to return to normal, presumably along the path described by the extrapolated broken line from three to five hours. The fact that the bicarbonate concentration in the anterior chamber fluid, instead of continuing to rise and return to normal levels, fell again to the previously reduced level clearly suggests that some factor other than the level in the posterior chamber is the major determinant of the level in the anterior chamber at this stage. The coincident drop of the bicarbonate concentration in the blood plasma starting after the third hour* and continuing to the end of the eighth appears to be the determinant condition for the alteration of the course of the bicarbonate concentration in the anterior chamber. This relationship constitutes an experimental confirmation of the postulate by Kinsey[12] that the bicarbonate ion in the aqueous humor of the anterior chamber of the rabbit eye is in passive diffusional exchange with the bicarbonate ion in the blood plasma.

Concerning the fall in bicarbonate concentration in the anterior chamber observed during the first two hours, it is important to note that this occurred while the bicarbonate concentration in the blood plasma had remained substantially unchanged. That this fall in the anterior chamber was probably not due to the previously mentioned phenomenon, namely, withdrawal of bicarbonate from the extravascular fluids to compensate for the Diamox-induced loss of the ion in the urine, is suggested by the tendency of the fluid in the anterior chamber to recover from its initial loss, even though short-lived under the experimental conditions.

Thus it would appear that the initial downward trend of the bicarbonate concentration in the aqueous humor of the anterior chamber following an intravenous injection of Diamox is a reflection of the changes occurring in the posterior chamber fluid, while the subsequent maintenance of the bicarbonate concentration at the reduced level (when the concentration in the posterior chamber has already returned to normal levels) is a direct reflection of the falling and reduced bicarbonate concentration in the arterial blood plasma.

The contributions of both factors at any time may be considered to be predominantly responsible for the maintenance of the level of bicarbonate concentration in the anterior chamber. The first factor is related to the active transport mechanism of bicarbonate ion into the posterior chamber, while the second factor is associated with the passive diffusion between the bicarbonate concentration in the aqueous humor of the anterior

* The slight rise in the curve during the fifth hour probably represents a "rebound" from the sharp drop in bicarbonate concentration during the previous hour.

chamber and that in the blood plasma.

It is appropriate at this time to call attention to the observation by Green and coworkers[2] that of all the carbonic anhydrase activity present in the anterior uvea of the rabbit eye, 40 percent was in the iris and 60 percent in the ciliary processes. Furthermore, it was found that the enzyme activity in both the iris and ciliary processes was completely inhibited in 30 minutes following a single intravenous injection of 30 mg. Diamox per kg. body weight.

Despite the theoretical implications of these related observations hardly any importance has been attached to the possible contribution of the enzyme in the iris to the bicarbonate concentration in the aqueous humor. Indeed, other than its participation in diffusional exchange of substances with the blood, the iris has not been considered to contribute to the elaboration of the bicarbonate ion in the aqueous humor.

Yet on morphologic and anatomic grounds, as well as the fact that carbonic anhydrase is present in the tissue, it is not unlikely that the iris also participates in the elaboration of the bicarbonate ion into the posterior chamber of the rabbit eye by an active transfer mechanism. Indeed, the results of this investigation, when viewed in the light of the previously mentioned studies of carbonic anhydrase in the iris, are compatible with the hypothesis that the decrease in bicarbonate concentration in the aqueous humor of the posterior chamber following an intravenous administration of Diamox is due to the inhibition of the enzyme activity in both the ciliary processes and iris.

Becker and Constant[6] have shown by repeated tonographic tracings on 50 rabbit eyes, that given a normal rate of aqueous secretion, a single intravenous injection of Diamox (100 mg./kg.) resulted in an average of 63-percent suppression of aqueous secretion in 45 to 60 minutes. They also pointed out that this estimate is in good agreement with measured alterations in fluorescein appearance time and is related to the fact that the bicarbonate concentration in the aqueous humor (posterior chamber) is decreased while that in the blood plasma remains constant (a decrease in the normal excess of bicarbonate concentration in the aqueous humor). A similar estimate of suppression of aqueous secretion was obtained under conditions of prolonged Diamox administration over a period of six hours, at which time the bicarbonate concentration in the aqueous humor of both chambers of the eye had fallen while the concentration in the blood plasma remained unchanged.

Langham and Lee,[10] on the other hand, maintain that the decrease in the excess bicarbonate in the aqueous humor of the anterior chamber (the posterior chamber was not analysed) over that in the arterial blood plasma is only transitory, since with repeated administration of Diamox over 2.5 hours, the bicarbonate concentration in the blood falls to the same extent as that in the aqueous humor. Similarly, they observed that the decrease in intraocular pressure, measured manometrically and tonometrically, as well as the increased fluorescein-appearance time were only transitory.

These investigators concluded, on the basis of their observations, that "the temporary reduction in the intraocular pressure, the rate of formation of aqueous humor, and the excess of bicarbonate in the aqueous humor immediately following the administration of Diamox are associated with the action of this substance in inducing a rapid fall in the pH of the aqueous humor." Furthermore, the continued presence of Diamox had little or no effect upon the intraocular dynamics of the normal rabbit eye.

It is apparent, therefore, that, while the data from Becker's laboratory do not agree with those from Langham's, in so far as the prolonged effects of Diamox are concerned, there is some concordance of results with the short-term effects of the drug. Thus, it is agreed that during the first 15 to 30 minutes following a single administration of Diamox, the intraocular pressure falls

significantly and the fluorescein appearance time increases. However, while Langham and Lee attributed this to the decrease in bicarbonate concentration in the anterior chamber fluid, with the blood bicarbonate remaining constant, Becker ascribed these phenomena to the decrease in the bicarbonate concentration in the posterior chamber fluid, with the blood bicarbonate remaining constant. On prolonged administration of Diamox Langham and Lee reported a return to normal of the intraocular dynamics, presumably because of a decrease in the blood bicarbonate concentration to the same extent as in the aqueous humor, while Becker observed a steady-state of reduced bicarbonate concentration in both the anterior and posterior chamber fluids, and no change in the blood concentration, with a sustained decreased aqueous secretion and intraocular pressure.

The fundamental chemical basis upon which the interpretation of the experimental results by the foregoing investigators rests is that the normal intraocular dynamics of the rabbit eye derives from the excess bicarbonate concentration in the aqueous humor over that in the blood plasma. Elimination or diminution of the excess is accompanied by a decrease in the rate of aqueous secretion and a drop in the intraocular pressure "responsive" animals. In this connection the results of the present investigation of the effects of a single administration of Diamox are of interest. The suppression of aqueous secretion that follows 45 to 60 minutes after the administration of Diamox correlates well with the observed decrease in the bicarbonate concentration in the aqueous humor, while the concentration in the plasma remains unaffected. (The rapid fall in bicarbonate concentration in the anterior chamber observed by Langham and Lee to occur in 15 minutes has not been confirmed.)

It was further observed by Becker and Constant, in a tonographic study of the effect of a single administration of Diamox upon aqueous flow, that restoration of the initial intraocular pressure in three to six hours was accomplished primarily by a decreased facility of outflow before aqueous flow had returned to normal. During this time period, as can be seen from Figure 1, the bicarbonate concentration of the aqueous humor in the posterior chamber returned to normal, the concentration in the blood plasma fell about 15 percent and the concentration in the anterior chamber fluid remained at the reduced level. In other words, the normal excess of bicarbonate concentration in the aqueous humor of the anterior chamber over that in the blood plasma was restored and the excess normally present in the posterior chamber fluid was increased by virtue of the decrease in bicarbonate concentration in the blood plasma.

On the basis of the ratio of the bicarbonate concentration in the aqueous humor of tween the aqueous humor and the blood plasma, the situation three to six hours after a single administration of Diamox may be regarded as normal, while the ratio of distribution between the fluid in the posterior chamber and that in the blood plasma is appreciably higher than the normal. Thus, if the rate of secretion of aqueous humor is fundamentally dependent upon the relative distribution of bicarbonate concentration between the aqueous humor and the blood plasma, then three to six hours after the intravenous injection of Diamox one should reasonably expect a state of normal intraocular dynamics, instead of the continued suppression of aqueous secretion as the tonographic data of Becker and Constant demonstrate. Furthermore, as referred to previously, the data of Green and co-workers showed that after the first hour following the intravenous administration of a single dose of Diamox (30 mg./kg.) the carbonic anhydrase activity of the anterior uvea tended to return to normal.

A reasonable explanation for the changes in the pH of the aqueous humor of the anterior chamber is based upon the consideration that the bicarbonate system is the main buffer responsible for the maintenance of a constant pH in this fluid. This being so then

it is not surprising that the curve describing the changes in pH of the aqueous humor is similar in form to that describing the changes in the bicarbonate concentration. However, it is particularly significant that the pH changes of the aqueous humor, at least up to the end of five hours, are too small to account for the magnitude of the changes in the bicarbonate concentration. These findings and conclusions are not in agreement with those of Langham and Lee who reported decreases of 0.15 and 0.09 pH units in the aqueous humor of rabbit eyes 15 minutes after a single dose of 50 mg./kg. and 20 mg./kg., respectively. These investigators concluded that the corresponding reductions of the bicarbonate concentration of 21.5 percent and 13.3 percent, respectively, were "associated with the action of Diamox in inducing a rapid fall in the pH of the aqueous humor."

The lack of quantitative correlation between the magnitudes of the changes in pH and bicarbonate concentration herein reported suggests that, following the action of Diamox at least, a mechanism, in addition to that of the buffering capacity of the bicarbonate system, may be operating to regulate the pH of the aqueous humor. Because of the theoretical implications of such a disparity, more experimental work should be carried out in order to establish the relation between the pH and the bicarbonate concentration of the aqueous humor. Indeed, it is interesting to speculate that such a mechanism may be primarily responsible for the maintenance of the relatively high alkalinity of the aqueous humor compared to the plasma.

The pH measurements of the arterial whole blood show that a single dose of Diamox (30 mg./kg.) had no apparent effect upon the hydrogen-ion concentration, at least for the first five hours. No particular significance can be attributed to the small drop during the sixth hour, since longer periods of time were not investigated. Suffice to say, however, that the constancy of the pH of the blood during the first three hours is consistent with the failure of Diamox to alter the bicarbonate concentration of the arterial plasma in this time interval. The absence of a drop in the pH after three hours, when the bicarbonate concentration was lowered, is suggestive of a state of "compensated acidosis." It is interesting, in this connection, to note that although Langham and Lee measured a significant reduction of 0.09 and 0.10 pH units in the pH of whole arterial blood 15 minutes following a single dose of 50 mg./kg. and 20 mg./kg., respectively, they concluded "from general physiologic principles it is unlikely that Diamox could cause a decrease in the pH and an increase in the $CO_2$ tension of the blood within 15 minutes." Accordingly, they suggested that the reduction in pH of the blood in the short time period reflected changes at the time of withdrawal. Since they attributed the transitory changes in aqueous humor secretion, intraocular pressure and bicarbonate concentration of aqueous humor to the rapid reduction of pH of the aqueous humor, their thesis is weakened by the apparently arbitrary evaluation of one set of results without a similar questioning of the pH data of blood of normal rabbits and after repeated administration of Diamox, since all the measurements were done in exactly the same manner.

On the basis of the results of this investigation and other published data already referred to, the following observations seem to be of fundamental importance with regard to the mechanism of action of Diamox upon the intraocular dynamics of the rabbit eye. The inhibition of carbonic anhydrase in the anterior uvea can explain the changes in the bicarbonate concentration of the aqueous humor and the short-term effects upon aqueous secretion following the intravenous injection of a single dose of Diamox. The continued suppression of aqueous secretion three to six hours after the injection of Diamox, when 50 percent of the carbonic anhydrase activity has been restored, is incompatible with the concept that the enzyme activity is responsible for the rate of secre-

tion of aqueous humor.

By the same token, since under these conditions the relative distribution of bicarbonate concentration between the aqueous humor of the anterior chamber and the blood plasma has returned to normal and that between the posterior chamber fluid and the blood plasma has exceeded the normal level, one can not state with certainty that the net flow of aqueous humor into the posterior chamber is primarily derived from the existence of an excess bicarbonate concentration in the aqueous humor relative to that in the blood. Furthermore, in view of the absence of any noticeable effect upon the bicarbonate concentration or the dynamics of the aqueous humor following inhibition of the carbonic anhydrase activity by the local application of Diamox, it is perhaps safe to conclude that the action of the drug upon the eye depends upon a sufficient concentration being present in the blood.

It is not presumed that these results or remarks define the mechanism of action of Diamox in suppressing aqueous humor secretion or can explain the compensatory adjustment of outflow mechanism following the use of the drug. However, it is considered possible that Diamox may be acting more directly upon the basic metabolic system responsible for a secretory mechanism and that the inhibition of carbonic anhydrase is of secondary importance.

## Summary

1. The effect of a single intravenous dose of Diamox (30 mg./kg.) to rabbits upon the bicarbonate-ion concentration in arterial plasma and the aqueous humors of the anterior and posterior chambers and upon the pH of the aqueous humor and whole blood was studied over varying periods of time.

2. During the first three hours the bicarbonate concentration of the plasma remained substantially unchanged, that in the aqueous humor of the posterior chamber fell about 20 percent and returned to its original normal value, and the bicarbonate concentration in the aqueous humor of the anterior chamber also fell about 20 percent, but more slowly than that in the posterior chamber, and had already started to return to its original normal value.

3. During the next interval of time the tendency of the bicarbonate concentration in the aqueous humor of the anterior chamber to recover from its 20-percent decrease after three hours was arrested and instead the concentration temporarily remained at the reduced level during the fourth hour and then again slowly fell to the level of a 20-percent reduction during the sixth hour, where it remained at least for an additional two hours.

4. The bicarbonate concentration of the aqueous humor of the posterior chamber returned to its original normal level in three hours and even exceeded the original concentration by about 12 percent during the fourth hour, but then slowly returned to the original level.

5. The bicarbonate concentration of the arterial blood plasma, after having remained constant for three hours, fell about 17 percent during the next three hours and remained at this reduced level for at least until the end of the eighth hour.

6. The time curve of the pH changes in the aqueous humor of the anterior chamber had the same form as time curve of the bicarbonate changes. The magnitude of the pH changes, however, could not account for the magnitude of the bicarbonate changes.

7. The pH of whole arterial blood was unchanged during the first five hours of the experiment then fell slightly during the sixth hour.

8. The results are discussed in the light of previously published results and evaluated with regard to the secretion-diffusion theory of aqueous humor secretion.

*1601 Spring Garden Street (30).*

### Acknowledgment

We wish to express our appreciation to Dr. I. H. Leopold, Director of Research, for his interest and helpful discussion and to Dr. S. I. Askovitz for helpful discussion.

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## Discussion

Dr. John E. Harris: This interesting paper by Dr. Green and his co-workers poses a lot of problems. I would like to discuss one problem that I think is most vexing, certainly the most vexing to Dr. Green, Dr. Becker, Dr. Langham, and myself, and that is the rather inconsistent data that have been obtained and reported in the literature. To add to the confusion, I would like to show some data that we have presented.

This shows the effects of Diamox on the total $CO_2$. Actually, it reflects pretty well the total bicarbonate. Again we have observed a fairly marked drop in the arterial plasma. This was fairly consistent, and was significant.

The total drop in $CO_2$ amounts to approximately 6 meq. per liter, which in the anterior chamber is approximately that which Dr. Green has shown. You will note that our steady state ratio remains fairly constant. That, of course, would not be true in Dr. Green's data.

Our data are fairly similar to those which Dr. Langham and Dr. Lee reported quite recently. It differs from those presented today. As a matter of fact, I think we are all right. The rabbit is a very capricious animal, and perhaps we are doing ourselves an injustice to fuss with the rabbit.

The inference from Dr. Green's studies would be that if Diamox is lowering the intraocular pressure, it is doing it in this manner: Bicarbonate is found in high concentration in the posterior chamber and in the anterior chamber. The secretion, active transfer (I think we will have to call it that definitely) —the active transfer of this material into the posterior chamber presumably creates a local hyperosmotic pressure with respect to plasma, and we get a flow of material.

If Diamox, then, depresses the formation of bicarbonate, then by inference the six meq. per liter drop would considerably reduce the osmotic pressure of the aqueous, and we would have a slowing of the aqueous flow. This would require, however, that we be able to demonstrate a drop in cation.

We have checked both sodium and potassium, in a certain sense anticipating the data we will present in just a moment, but in no instance have we found any preferential drop in sodium or potassium. The drop in hydrogen-ion concentration, while significant, is not a sufficient osmotic force to balance the bicarbonate.

The other choice is that there has been a shift in chloride, and this perhaps would explain all of our data, that under certain circumstances we are measuring different things at different times.

This would not in any sense rule out the possibility, however, that Diamox does act as a carbonic anhydrase inhibitor—that it does alter the bicarbonate concentration. This we know. The question is whether it alters it preferentially with respect to plasma.

I think, therefore, the present theory by which we get this bicarbonate into the anterior chamber or into the posterior chamber is certainly adequate.

I would agree with Dr. Green that probably the total effects of Diamox, however, are not to be attributed solely to this mechanism. I think the best thing we can say about Diamox is that it sure works in a peculiar manner.

Dr. Harry Green (closing): I should like to thank Dr. Harris for his pertinent and cogent remarks, and for his contribution to the elucidation of the mechanism of action of Diamox in suppressing aqueous secretion.

Admittedly, there is a variety of speculations one can introduce at this time. However, I feel that there is increasing agreement that Diamox may not be working directly upon carbonic anhydrase in the anterior uvea, but may be affecting some other fundamental metabolic system or systems basically involved in the generation of energy for the maintenance of the active transfer mechanisms.

If active transfer mechanisms are operating in the eye, especially in the posterior chamber, then we should attempt to uncover the metabolic or physicochemical nature of the processes.

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# THE BICARBONATE CONCENTRATION OF GUINEA PIG AQUEOUS HUMOR*

## Effect of scurvy and acetazolamide

Bernard Becker, M.D.
*Saint Louis, Missouri*

In recent years the possible roles of bicarbonate and ascorbate in the secretion of the aqueous humor have aroused considerable interest and speculation. Although in the acid secretion of the human eye, bicarbonate is actually in deficit of plasma values,[1,2] the excesses of bicarbonate in the aqueous humor of rabbits and guinea pigs are most striking. Thus, Friedenwald and Buschke[3] postulated for rabbits and guinea pigs that bicarbonate was a consequence of the production of hydroxyl ions by the redox pump, and that ascorbate was a mediator in the chain of energy for this secretion. The accumulation of intravenously administered crystal violet in the epithelium of the guinea pig ciliary processes was found to be slowed down by dietary deficiencies of vitamin C. Furthermore, scorbutic guinea pigs demonstrated a delay in restoration of the anterior chamber after withdrawal of aqueous humor. Both of these effects were promptly reversible by the administration of ascorbic acid. The finding of Scholz[4] that the large excess of sodium in the guinea pig anterior chamber aqueous decreased to dialysate levels in the scorbutic animal tended to confirm the Friedenwald hypothesis. However, using the para-amino hippurate method, Bárány[5] could detect no induced alterations in flow rates in guinea pigs by ascorbic acid deficiency or saturation. More recently Ridge[6] re-studied the chloride and sodium concentrations in the anterior chamber aqueous of guinea pigs. He noted no significant differences in sodium excess or chloride deficit in the anterior chamber of scorbutic or vitamin-C saturated animals.

In the rabbit the large bicarbonate excess of the anterior chamber aqueous and even higher concentration in the posterior chamber are reduced significantly when the secretion of aqueous humor is partially suppressed by acetazolamide. Furthermore, using a simplified form of the Kinsey equation,[7] estimates of the induced alterations in the ratio of flow rate to diffusion rate could be obtained from the changes in steady state concentrations of bicarbonate in the plasma, posterior chamber, and anterior chamber.[8] The values so derived were in reasonable agreement with the results of turnover studies[9] and repeated tonographic measurements.[10]

The purpose of the present work was threefold. First to study the concentration

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* From the Department of Ophthalmology, Washington University School of Medicine and the Oscar Johnson Institute, Saint Louis.

This investigation was supported in part by a research grant, B-621, from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

The research relating to this study was also financed in part under a grant to Washington University School of Medicine made by the Alfred P. Sloan Foundation, Inc. The grant was made upon recommendation of the Council for Research in Glaucoma and Allied Diseases. Neither the Foundation nor the Council assumes any responsibility for the published findings of this study.

of bicarbonate in posterior chamber as well as anterior chamber of a species other than the rabbit. Secondly, to study the role of ascorbate in maintaining the bicarbonate excess in the aqueous humor of the guinea pig. Lastly, to attempt to alter the concentration of bicarbonate by acetazolamide administration. With posterior chamber aqueous values it was hoped to detect and estimate alterations in flow rate not revealed by anterior chamber and plasma concentrations alone.

## Methods

Guinea pigs weighing 500 to 800 gm. were obtained from Haskins Rabbitry. "Normal" animals were kept on a diet of Purina rabbit chow ad libitum with daily liberal supplements of lettuce. The "scorbutic" animals were maintained on a mixture of: bran 24 percent, barley meal 16 percent, wheatlings 21 percent, fish meal 8.0 percent, crushed oats 26 percent, salt mixture 5.0 percent, and reconstituted whole milk plus 1.0 cc. of 0.5-percent copper sulfate per 100 cc. of milk.[11] "Scorbutic controls" were on the same diet as scorbutic animals but received daily subcutaneous injections of 50 mg. sodium ascorbate.

After a period of 12 to 22 days on the scorbutic diet, animals were found to have less than 0.05 mM/kg. water ascorbate in their aqueous humor, to lose weight, and to begin to develop hemorrhagic lesions. Scorbutic controls supplemented with ascorbate remained healthy and had anterior chamber ascorbate levels of 1.0 to 1.5 mM/kg. water. All animals were kept quietly in the room of the experiment in individual cages for 24 to 48 hours before obtaining samples. Unanesthetized animals were used throughout and all animals were discarded after single taps.

Blood was obtained by cardiac puncture using a heparinized syringe. It was introduced directly beneath USP paraffin oil and centrifuged at once in the cold. Posterior chamber and anterior chamber aqueous were drawn from the tetracaine-anesthetized proptosed eyes into calibrated pipets: 10 to 15 µl. were obtained from the posterior chamber; 50 to 60 µl. from anterior chamber taps. These samples were introduced directly into the Van Slyke apparatus for the determination of total carbon dioxide.

Bicarbonate values were obtained by correcting for carbon dioxide using the average pH values found for normal guinea pig plasma (7.38) and aqueous humor (7.50); and acetazolamide-treated values of 7.33 (plasma) and 7.41 (aqueous). Ascorbate was determined by titration with dichlorphenol-indophenol in four-percent metaphosphoric acid. Ascorbate or acetazolamide were administered as the isotonic solution of the sodium salt by intraperitoneal injection. The acetazolamide dose was 70 mg./kg. initially followed by 35 mg./kg. every hour for six hours.

## Results

The concentration of bicarbonate in the posterior chamber aqueous was found to average 37.5 mM/kg. water or an excess of 54 percent over the plasma value. The mean anterior chamber aqueous humor bicarbonate level was 34.8 mM or 43 percent in excess of plasma (table 1). Thus, although

TABLE 1

AQUEOUS BICARBONATE OF GUINEA PIGS (MM/KG. WATER): (NORMAL DIET)

| | $C_{PC}$ | $C_{AC}$ | $C_{Pl}$ | $\frac{K_F}{K_D}$ |
|---|---|---|---|---|
| | 31.9 | 30.1 | 24.3 | 3.2 |
| | 38.4 | 35.7 | 26.7 | 3.2 |
| | 38.0 | 34.3 | 22.1 | 3.3 |
| | 32.4 | 30.9 | 34.1 | 4.5 |
| | 38.8 | 35.9 | 25.5 | 3.6 |
| | 35.1 | 32.4 | 24.0 | 3.1 |
| | 39.2 | 36.0 | 23.7 | 3.8 |
| | 39.4 | 35.8 | 23.8 | 3.3 |
| | 40.7 | 38.5 | 26.2 | 5.6 |
| | 39.8 | 37.8 | 26.2 | 5.8 |
| | 38.5 | 35.1 | 21.3 | 4.1 |
| Mean | 37.5 | 34.8 | 24.4 | 4.0 |
| S.D. | | | | ±0.9 |

$C_{PC}$ = Concentration in posterior chamber aqueous.
$C_{AC}$ = Concentration in anterior chamber aqueous.
$C_{Pl}$ = Concentration in plasma.
$K_F$ = Coefficient of anterior chamber aqueous flow.
$K_D$ = Coefficient of diffusion of bicarbonate between plasma and anterior chamber.

TABLE 2

AQUEOUS BICARBONATE OF GUINEA PIGS (MM/KG. WATER): (SCORBUTIC DIET)

| | $C_{PC}$ | $C_{AC}$ | $C_{PI}$ | $\frac{K_F}{K_D}$ |
|---|---|---|---|---|
| | 40.3 | 37.4 | 24.4 | 4.5 |
| | 39.9 | 37.1 | 25.3 | 4.2 |
| | 36.3 | 34.7 | 26.4 | 5.2 |
| | 40.5 | 38.5 | 27.7 | 5.4 |
| | 33.3 | 31.4 | 22.9 | 4.5 |
| | 32.8 | 31.3 | 23.8 | 5.0 |
| | 31.1 | 29.3 | 21.6 | 4.2 |
| | 29.9 | 28.0 | 19.7 | 4.4 |
| | 35.2 | 33.5 | 25.4 | 4.8 |
| | 38.1 | 35.3 | 24.8 | 3.8 |
| | 40.2 | 36.8 | 23.4 | 3.9 |
| | 36.9 | 34.1 | 21.2 | 3.4 |
| | 34.9 | 32.2 | 25.8 | 2.9 |
| | 34.8 | 32.5 | 22.8 | 4.2 |
| | 33.3 | 31.3 | 23.5 | 3.9 |
| | 37.8 | 34.2 | 21.3 | 3.6 |
| | 38.5 | 35.7 | 24.2 | 4.1 |
| | 37.9 | 35.3 | 24.1 | 4.3 |
| Mean | 36.2 | 33.8 | 23.8 | 4.2 |
| S.D. | | | | ±0.6 |

$C_{PC}$ = Concentration in posterior chamber aqueous.
$C_{AC}$ = Concentration in anterior chamber aqueous.
$C_{PI}$ = Concentration in plasma.
$K_F$ = Coefficient of anterior chamber aqueous flow.
$K_D$ = Coefficient of diffusion of bicarbonate between plasma and anterior chamber.

the posterior chamber concentration in rabbits and guinea pigs are quite comparable, the anterior chamber of the guinea pig retains a considerably greater excess of this ion than does the rabbit eye. Since the flow rate constant $K_F$ is probably much the same in both species, there is likely a decreased rate of diffusional exchange of the bicarbonate ion in the guinea pig anterior chamber.

In animals on the scorbutic diet no significant difference in bicarbonate concentrations could be detected between the scorbutic group (table 2) and the vitamin C-supplemented controls (table 3), nor did either group differ from the normal animals (table 1). Thus, the average value (±S.D.) for the $K_F/K_D$ of bicarbonate in the normal animals was 4.0 ± 0.9 while for the scorbutic group the mean (±S.D.) was 4.2 ± 0.6 and for the scorbutic control 3.9 ± 0.8. Furthermore, when scorbutic animals were given 50 mg. of ascorbate after withdrawing the initial samples from one eye, no significant changes in bicarbonate concentration in posterior chamber or anterior chamber aqueous could be detected after six hours (table 4). This failure to alter bicarbonate concentrations or the ratio $K_F/K_D$ occurred in spite of a measured change in anterior chamber aqueous ascorbate in the same scorbutic animals from less than 0.05 in the first eye to over 1.0 mM/kg. water in the second eye. These data provide very suggestive evidence that ascorbate is neither a necessity for maintaining the bicarbonate excess in the aqueous humor nor an important factor in determining the rate of aqueous flow.

The administration of acetazolamide to normal, scorbutic, or vitamin C-saturated guinea pigs resulted in comparable impressive falls in the bicarbonate excesses of both posterior chamber and anterior chamber aqueous (table 5). The posterior chamber excess over plasma was reduced from an average of 12.3 mM (52 percent) to 6.0 mM (30 percent) and anterior chamber values from 9.8 mM (41 percent) greater than plasma to 3.8 mM (19 percent). This reduc-

TABLE 3

AQUEOUS BICARBONATE OF GUINEA PIGS (MM/KG. WATER): (SCORBUTIC DIET PLUS VITAMIN C SUPPLEMENT)

| | $C_{PC}$ | $C_{AC}$ | $C_{PI}$ | $\frac{K_F}{K_D}$ |
|---|---|---|---|---|
| | 37.7 | 34.5 | 24.5 | 3.1 |
| | 39.6 | 37.0 | 24.2 | 4.9 |
| | 39.2 | 37.0 | 26.4 | 4.8 |
| | 45.7 | 41.9 | 26.0 | 4.2 |
| | 35.2 | 33.5 | 24.8 | 5.1 |
| | 31.3 | 28.9 | 20.0 | 3.7 |
| | 38.2 | 34.0 | 22.3 | 2.8 |
| | 34.7 | 32.1 | 24.6 | 2.9 |
| | 35.3 | 33.0 | 24.6 | 3.7 |
| Mean | 37.4 | 34.7 | 24.2 | 3.9 |
| S.D. | | | | ±0.8 |

$C_{PC}$ = Concentration in posterior chamber aqueous.
$C_{AC}$ = Concentration in anterior chamber aqueous.
$C_{PI}$ = Concentration in plasma.
$K_F$ = Coefficient of anterior chamber aqueous flow.
$K_D$ = Coefficient of diffusion of bicarbonate between plasma and anterior chamber.

TABLE 4

THE EFFECT OF ASCORBATE ADMINISTRATION ON THE AQUEOUS HUMOR BICARBONATE CONCENTRATION (MM/KG. WATER) OF SCORBUTIC GUINEA PIGS

| | Before | | | | 6 hours after Ascorbate (50 mg. intraperitoneal) | | | |
|---|---|---|---|---|---|---|---|---|
| | $C_{PC}$ | $C_{AC}$ | $C_{PI}$ | $\frac{K_F}{K_D}$ | $C_{PC}$ | $C_{AC}$ | $C_{PI}$ | $\frac{K_F}{K_D}$ |
| | 35.2 | 33.5 | 25.4 | 4.8 | 36.0 | 33.6 | 24.2 | 3.9 |
| | 38.1 | 35.3 | 24.8 | 3.8 | 38.4 | 35.2 | 24.3 | 3.4 |
| | 40.2 | 36.8 | 23.4 | 3.9 | 39.8 | 36.7 | 24.9 | 3.7 |
| | 36.9 | 34.1 | 21.2 | 3.4 | 37.7 | 34.6 | 21.6 | 4.2 |
| | 34.9 | 32.2 | 25.8 | 2.9 | 34.5 | 32.5 | 25.0 | 2.5 |
| | 34.8 | 32.5 | 22.8 | 4.2 | 33.6 | 31.8 | 23.6 | 4.5 |
| | 33.3 | 31.3 | 23.5 | 3.9 | 33.5 | 30.9 | 21.9 | 3.6 |
| | 37.8 | 34.2 | 21.3 | 3.6 | 40.0 | 36.6 | 24.0 | 3.7 |
| | 38.5 | 35.7 | 24.2 | 4.1 | 37.5 | 35.4 | 25.5 | 4.7 |
| | 37.9 | 35.3 | 24.1 | 4.3 | 35.0 | 32.9 | 24.6 | 4.0 |
| Mean | 36.8 | 34.1 | 23.7 | 3.9 | 36.6 | 34.0 | 24.0 | 3.8 |
| S.D. | | | | ±0.5 | | | | ±0.6 |

$C_{PC}$ = Concentration in posterior chamber aqueous.
$C_{AC}$ = Concentration in anterior chamber aqueous.
$C_{PI}$ = Concentration in plasma.
$K_F$ = Coefficient of anterior chamber aqueous flow.
$K_D$ = Coefficient of diffusion of bicarbonate between plasma and anterior chamber.

tion in bicarbonate concentrations was thus accomplished in a fashion consistent with an average decrease in the $K_F/K_D$ ratio of 58 percent.

## DISCUSSION

The failure to alter posterior chamber or anterior chamber aqueous bicarbonate levels in spite of enormous variations in ascorbate levels is consistent with the lack of change in anterior chamber concentrations of sodium and chloride. The data fail to support the role attributed to ascorbate in production of bicarbonate in the aqueous. Thus, in deficient animals aqueous ascorbate was less than 0.05 mM/kg. water, whereas normal animals averaged 0.55 mM/kg. water, and the ascorbate saturated controls had values of 1.0 to 1.5 mM/kg. In all three of these series there was no detectable change in the ratio $K_F/K_D$. This agrees well with Bárány's para-amino hippurate data which indicate no alteration in $K_F$ under similar circumstances. These chemical findings are also confirmed by entirely independent physical methods of measurement.[12] Thus, in vivo perfusion studies indicate that the intraocular pressure and outflow facility do not differ in scorbutic and control animals. Furthermore, ascorbate administration to scorbutic animals fails to alter either intraocular pressure or perfusion outflow. In fact, the lack of change in flow rate makes it apparent that the diffusion constant $K_D$ for bicarbonate is also altered very little by variation in ascorbate level.

The effects of acetazolamide on the guinea pig eye are quite comparable with the rabbit data. Perfussion data in guinea pigs also indicate that the fall in intraocular pressure occurs without alteration in outflow facility in this species as in the rabbit and human eye.[12] These results suggest the need for carbonic anhydrase in order to maintain the bicarbonate excess of guinea pig aqueous, much as in the rabbit. It is of further interest that the decrease in bicarbonate excess in the posterior chamber from 12.3 mM to 6.0 mM is of the same order of magnitude as the decrease in rate of aqueous secretion. This does not necessarily signify, however, that bicarbonate is the primary secretory product. It merely means that bicarbonate formation is reduced to a greater extent than

TABLE 5

AQUEOUS BICARBONATE OF GUINEA PIGS (mM/KG. WATER): EFFECTS OF ACETAZOLAMIDE

| | Before Acetazolamide | | | | 6 Hours After Acetazolamide* | | | |
|---|---|---|---|---|---|---|---|---|
| | $C_{PC}$ | $C_{AC}$ | $C_{PI}$ | $\frac{K_F}{K_D}$ | $C_{PC}$ | $C_{AC}$ | $C_{PI}$ | $\frac{K_F}{K_D}$ |
| | 38.8 | 35.9 | 25.5 | 3.6 | 28.8 | 25.4 | 20.1 | 1.6 |
| | 35.1 | 32.4 | 24.0 | 3.1 | 27.1 | 24.4 | 21.0 | 1.3 |
| | 39.2 | 36.0 | 23.7 | 3.8 | 28.3 | 26.1 | 22.7 | 1.6 |
| | 39.4 | 35.8 | 23.8 | 3.3 | 27.3 | 23.7 | 19.8 | 1.1 |
| | 38.5 | 35.1 | 21.3 | 4.1 | 26.9 | 23.5 | 18.8 | 1.4 |
| | 31.9 | 30.1 | 24.3 | 3.2 | 26.0 | 24.6 | 21.2 | 2.4 |
| | 38.4 | 35.7 | 26.7 | 3.2 | 25.0 | 23.1 | 19.8 | 1.7 |
| | 38.0 | 34.3 | 22.1 | 3.3 | 23.5 | 20.6 | 17.3 | 1.1 |
| | 32.4 | 30.9 | 24.1 | 4.5 | 23.6 | 21.9 | 18.5 | 2.0 |
| a | 31.1 | 29.3 | 21.6 | 4.2 | 26.8 | 23.8 | 16.5 | 2.4 |
| a | 29.9 | 28.0 | 19.7 | 4.4 | 23.8 | 21.8 | 19.2 | 1.3 |
| a | 32.8 | 31.3 | 23.8 | 5.0 | 25.5 | 24.1 | 21.0 | 2.2 |
| a | 33.3 | 31.4 | 22.9 | 4.5 | 24.6 | 22.9 | 20.1 | 1.7 |
| a | 40.5 | 38.5 | 27.7 | 5.4 | 30.9 | 28.0 | 22.0 | 1.7 |
| a | 36.3 | 34.7 | 26.4 | 5.2 | 25.5 | 24.1 | 21.4 | 1.9 |
| a | 39.9 | 37.1 | 25.3 | 4.2 | 24.0 | 23.3 | 22.4 | 1.3 |
| a | 40.3 | 37.4 | 24.4 | 4.5 | 25.7 | 25.0 | 23.8 | 1.7 |
| b | 38.2 | 34.0 | 22.3 | 2.8 | 27.2 | 24.8 | 21.6 | 1.3 |
| b | 31.3 | 28.9 | 20.0 | 3.7 | 26.0 | 23.5 | 19.7 | 1.5 |
| b | 35.2 | 33.5 | 24.8 | 5.1 | 28.0 | 25.0 | 18.0 | 2.3 |
| Mean | 36.0 | 33.5 | 23.7 | 4.1 | 26.2 | 24.0 | 20.2 | 1.7 |
| S.D. | | | | ±0.8 | | | | ±0.4 |

a = Scorbutic animals.
b = Scorbutic diet plus Vitamin C supplement.
* = Isotonic solution of sodium salt of acetazolamide by intraperitoneal injection (70 mg/Kg initially then 35 mg/Kg every hour).
† $(K_F/K_D) = 12$ (before and after acetazolamide).
$C_{PC}$ = Concentration in posterior chamber aqueous.
$C_{AC}$ = Concentration in anterior chamber aqueous.
$C_{PI}$ = Concentration in plasma.
$K_F$ = Coefficient of anterior chamber aqueous flow.
$K_D$ = Coefficient of diffusion of bicarbonate between plasma and anterior chamber.

the entry of water into the posterior chamber aqueous humor following carbonic anhydrase inhibition. Furthermore, acetazolamide administration is capable of altering many secretions directly or indirectly dependent upon carbonic anhydrase and the buffering capacity it offers the secretory cell. Thus, the acid secretion of the gastric mucosa[13] and the human ciliary body[2] as well as the alkaline products of the pancreas[14] and rabbit and guinea pig ciliary body are all reduced by carbonic anhydrase inhibitors.

## SUMMARY

1. The concentrations of bicarbonate in the posterior chamber, anterior chamber, and plasma of guinea pigs are compared. Average values found for normal animals are 37.5, 34.8, and 24.4 mM/kg. water.

2. In spite of enormous differences in anterior chamber ascorbate levels between scorbutic and vitamin C-supplemented guinea pigs, no significant differences in concentrations of bicarbonate in the anterior or posterior chambers or in the ratio $K_F/K_D$ are apparent. Furthermore, ascorbate administration to deficient animals does not alter aqueous bicarbonate concentrations in either chamber in the same animal after six hours. Thus, neither the rate of flow of aqueous humor nor its excess of bicarbonate appear to depend upon ascorbate levels.

3. Acetazolamide administration decreases the bicarbonate excess over plasma in the

posterior and anterior chambers. Values obtained for posterior chamber (26.2 mM/kg. water), anterior chamber (24.0 mM/kg. water) and plasma (20.2 mM/kg. water) after systemic carbonic anhydrase inhibition are compatible with an average of 58-percent suppression of aqueous flow.

*640 South Kingshighway (10).*

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## Discussion

Dr. Harry Green (Philadelphia): While it is generally believed that ascorbic acid enters the posterior chamber of the animal eye as a result of an active transfer mechanism, the experimental evidence of Dr. Becker, gathered by well-accepted procedures, support the conclusions of Bárány that ascorbic acid does not play an important role in the sequence of events associated with the secretion of aqueous humor. Dr. Becker has demonstrated further that ascorbic acid apparently is not necessary for the elaboration or maintenance of the bicarbonate concentration in the aqueous humor of the guinea pig.

Although the precise role that ascorbic plays in mammalian tissue and the intraocular fluids is not clear, of immediate interest to this group, perhaps, is information as to the mechanism of its elaboration in the aqueous humor. In this regard, I feel that Dr. Becker's previously published data (Am. J. Ophth., **40**:129 [Nov. Pt. II] 1955) on the effect of Diamox upon the bicarbonate and ascorbate concentrations in the aqueous humor of the rabbit eye are of interest. At that time he showed that concomitant with the Diamox-induced suppression of aqueous secretion and the reduction in the concentration of bicarbonate in the rabbit eye the concentration of ascorbic acid in the posterior chamber fluid increased by 50 percent.

In view of the fact that the amount of increase could have been as much as 150 percent, if ascorbic acid secretion were not inhibited at all, with the water inflow depressed 60 percent, Dr. Becker concluded "that the entrance of ascorbic acid into the eye is partially inhibited by acetazolamide in spite of the fact that its concentration rises."

At the same time he suggested as a possible explanation for the increase in ascorbic acid in the aqueous humor that the Vitamin may be actively transferred into the posterior chamber by a related or independent mechanism that is less dependent on carbonic anhydrase activity than aqueous secretion and the elaboration of the bicarbonate ion.

Thus the evidence suggests that while the elaboration of the bicarbonate ion into the aqueous humor may be dependent upon the carbonic anhydrase activity of the anterior uvea, the active transport of

ascorbate is probably not. The fact that Diamox inhibited both mechanisms suggests that the drug may also be acting upon an enzyme system other than that of the carbonic anhydrase. Indeed, it is reasonable to assume that Diamox may be acting directly upon a sequence of metabolic steps which regulate both mechanisms as well as the aqueous secretion and that the inhibition of carbonic anhydrase is only of secondary importance.

DR. GREEN (continuing): I should like to say, with regard to the distribution of bicarbonate-ion concentration in the aqueous humor and plasma of animals other than the rabbit and guinea pig, specifically man, the data in our laboratory suggest possibly that the distribution of the bicarbonate between the aqueous humor of man and that of blood plasma may not be as indicated by Dr. Becker and Dr. Davson.

Indeed, we have found that in the cataractous eyes the ratio of the bicarbonate in the aqueous humor to that in blood plasma is low, approximately 13 percent lower than that in the plasma. However, in those eyes which contain a posterior segment tumor, the bicarbonate concentration in the aqueous humor of the anterior chamber is significantly higher than that in the plasma.

Furthermore, in three cases of central retinal occlusion the bicarbonate concentration of the aqueous humor, if based upon the arterial blood plasma, is about equal to that of the plasma, giving a ratio of about one, or slightly less.

Furthermore, in two normal eyes with amblyopia in which the aqueous humor is apparently normal, the bicarbonate concentration of the aqueous humor is less than five percent lower than that in the venous plasma and, indeed, would probably be higher than that of the arterial plasma.

So, before we can draw any specific conclusions regarding the distribution of the bicarbonate-ion concentration between the aqueous humor and plasma of man, it is necessary to get a significant number of analyses from what may be considered to be normal aqueous humor.

DR. BERNARD BECKER (closing): I would like to thank Dr. Green for his discussion. I differ with him in nothing that he has said. However, I would like to add that in my opinion the simplest working hypothesis ought to be considered until it is disproved.

If we assume for the secretion of bicarbonate, ascorbic, sodium or any other ion into the eye of man, rabbit or guinea pig that the role of a buffer is necessary for the constant function of the secretory cell, then we have a role for carbonic anhydrase as a means of potentiating secretion. Furthermore, if we subject the cell of the ciliary epithelium to uncontrolled acidosis or alkalosis, inhibiting or preventing adequate buffering capacity by such agents as Diamox will decrease its capacity to secrete whatever it is secreting. This will include bicarbonate, ascorbate, and anything else that it does secrete.

On the other hand, there is no need to assume that the inhibition of ascorbate secretion must be to the same extent as that of bicarbonate or water. The secretion of these might be accomplished in entirely different fashions by the cell.

Finally, I would like to point out that it is an oversimplication to speak of the ratio of the anterior chamber to plasma concentrations as unity. We are disregarding the Donnon factor, you must remember, and there ought to be an excess of some four to five percent of anions in the anterior chamber. If one confines one's thinking to bicarbonate, it thus becomes evident that data showing a ratio of one or a little over one could still represent a deficit as compared with the Donnon equilibrium. Our data all happened to fall in the group with a small actual deficit of bicarbonate.

# THE AQUEOUS:SERUM SODIUM AND POTASSIUM STEADY-STATE RATIOS IN THE RABBIT*

## AND THE INFLUENCE OF DIAMOX AND DIBENAMINE THEREON

JOHN E. HARRIS, M.D., A. EUGENE CARLSON, M.D., LOUISE GRUBER, B.S., AND GERTRUDE HOSKINSON, B.A.
*Portland, Oregon*

In recent years analytic studies of the aqueous as compared to the plasma under steady-state and other conditions have been concerned largely with the detailed determination of anions rather than cations. A major reason for this emphasis undoubtedly arises from the fact that more striking deviations from the Donnan equilibrium have been observed in anions such as ascorbate, lactate, bicarbonate and chloride. Most interest has been centered around bicarbonate which, in the rabbit, was found to be present in the aqueous in higher concentration than in the plasma.[1] It was thus suggested that bicarbonate is the ion primarily transported into the aqueous of this animal, the cations following only secondarily as necessary to maintain electrical neutrality. The conclusion was of particular significance since it dovetailed with the theory of Friedenwald[2] that the active process responsible for aqueous formation stemmed from metabolic activity occurring across the ciliary epithelium and resulted in an accumulation of bicarbonate in the aqueous. The concept received further support when a carbonic anhydrase inhibitor was found to lower the ocular tension.[3,4]

In recent studies, however, Davson and Luck[5] found a marked species difference in the aqueous: plasma bicarbonate steady-state ratios. In certain animals the aqueous: plasma bicarbonate ratio was seen to be well below that predicted by the Donnan equilibrium. In these animals the authors found the chloride ratio to be relatively high. Extending their studies Davson, et al.[6] have shown that the aqueous:plasma sodium ratio generally runs higher than would be predicted from a Donnan equilibrium and have concluded therefrom that a cation is the major ion secreted or actively transported from the plasma into the aqueous humor. However, the observation that the sodium concentration of the aqueous is higher than that predicted from a Donnan equilibrium cannot be taken as prima facie evidence that a cation rather than an anion is primarily transported. The existing data could as readily indicate a primary anion transport, in some animals, chloride, and in others, bicarbonate, the difference depending upon the direction in which the oxidation-reduction potentials are oriented.

Of the cations, the aqueous:plasma steady-state ratios of both sodium and potassium have been frequently measured. For the most part the values reported in the literature have been obtained by chemical analysis and more recently by measurement of the distribution of radio-active ions. Surprisingly little study has been made of the simultaneous concentration of both sodium and potassium in one aqueous sample. Such studies as have been reported have been made generally on pooled samples or by older methods and have generally concluded that the steady-state ratios of sodium and potassium are substantially the same.[1,7] Such measurements could be of considerable importance, however, in resolving the question of whether an anion or cation is primarily transferred. If only an anion were actively transported, sodium and potassium should show the same aqueous:plasma ratio. (It can be considered that the mobility of the ion would not influence its movement in re-

* From the John E. Weeks Memorial Laboratory, Department of Ophthalmology, University of Oregon Medical School. Supported in part by Grant B-187 of the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda 14, Maryland.

sponse to an electrical charge. Thus, in this instance the fact that sodium is heavily hydrated and has a slower mobility than potassium is not of significance.)

With the development of flame photometry during the past 10 years it has become possible to measure sodium and potassium in the same aqueous sample. Two years ago while doing some studies on the cornea of the rabbit[8] we had occasion to analyze the aqueous by such technique and found the aqueous:serum potassium steady-state ratio to be uniformly and significantly higher than that of sodium. The present report is an expansion of those studies.

## Methods

Steady-state ratios were determined on samples of aqueous and serum* drawn as near simultaneously as possible. No anesthesia was employed except that the cornea was anesthetized with 0.5-percent tetracaine prior to withdrawing the aqueous. Generally 0.15 cc. of anterior chamber aqueous was drawn from all animals tested. In the rabbit and cat arterial serum obtained by heart puncture was analyzed. In the monkey and human venous blood was generally obtained from the forelimb. It is to be emphasized that heart puncture blood although presumably arterial may contain venous blood as well. In reality, under steady-state conditions, the difference between arterial and venous content of the cations is nil and can be ignored. Sodium and potassium were determined using a Baird Associates flame photometer employing the internal standard. With this technique the error of analysis can be taken conservatively as no greater than two percent. Calculations are presented as mEq./1,000 gm. of water accepting a value of 93 percent water for the serum and 99 percent water for the aqueous.†

In certain instances the effect of various drugs on the sodium and potassium concentration of the serum and aqueous humor of the rabbit was determined. In these instances the aqueous of one eye was withdrawn and a heart puncture performed. After an appropriate time interval the drug was injected and aqueous from the other eye together with another blood sample then taken at the indicated time.‡ In this manner comparison was always made between the aqueous samples of the two eyes of the same animal it having been previously determined that the variation from eye to eye in the same animal was considerably less than between the eyes of different animals.

## Results

The steady-state ratios of four species have been measured. In the rabbit it was observed that the aqueous:serum ratio for potassium generally was in excess of one (table 1). A steady-state ratio of less than one was rarely observed and when seen was generally in the high 0.90's. Contrariwise, the sodium steady-state ratios were uniformly lower. It should be emphasized that in general the variation from animal to animal of the sodium ratio was considerably less than that of potassium, probably due to the fact that the serum potassium level is much more labile than is that of sodium.

When other species were studied, however, it was found that this distribution of

* Serum was analyzed rather than plasma so as to avoid an additive anticoagulant. The values can probably be used interchangeably. In a control series, the cation values of serum and heparinized plasma of the same animal were compared, making allowance for sodium added with the anticoagulant. The potassium values of serum and plasma were identical while the sodium of plasma was slightly lower than that of serum.

† The serum water value represents a reasonable average of those obtained in this laboratory for the rabbit over several years. The error accepted by assuming a value is small and does not warrant determination of water content in every animal.

‡ In conducting these experiments at least three hours were allowed to elapse between the drawing of the control and the test blood and aqueous samples. A control series had indicated that the manipulation attending heart puncture caused a decrease in serum potassium. With three hours interval between heart punctures the difference in potassium was not sufficient to be significant.

TABLE 1

SODIUM AND POTASSIUM CONCENTRATIONS OF THE SERUM AND AQUEOUS OF VARIOUS SPECIES

| Animal | Serum | | Aqueous | | Ratio | |
|---|---|---|---|---|---|---|
| | mEq/1000 gm. Water | | mEq/1000 gm. Water | | Aqueous/Serum | |
| | Potassium | Sodium | Potassium | Sodium | Potassium | Sodium |
| Rabbit | (29) | (29) | (41) | (38) | | |
| | 4.27 | 150.3 | 4.40 | 140.6 | 1.03 | 0.94 |
| S.D. | ±0.3 | ±1.7 | ±0.2 | ±1.8 | | |
| Monkey | (11) | (11) | (25) | (25) | | |
| | 4.50 | 160.3 | 4.26 | 155.6 | 0.95 | 0.97 |
| S.D. | ±0.3 | ±3.2 | ±0.1 | ±1.8 | | |
| Cat | (12) | (12) | (23) | (23) | | |
| | 4.99 | 163.5 | 4.31 | 159.3 | 0.86 | 0.97 |
| S.D. | ±0.5 | ±1.9 | ±0.2 | ±2.4 | | |

Number of observations is given in parenthesis.

potassium was not uniformly observed. In the monkey the sodium and the potassium ratios were both found to be of the same magnitude, that is, 0.95 for potassium and 0.97 for sodium. In the cat, however, the potassium ratios were generally in the 0.80's. By contrast the sodium ratio was substantially that seen in the monkey, namely 0.97.

A few human eyes were also examined, the aqueous being taken at the time of surgery (table 2). Only one of these can be considered to have been a normal eye. In this case the globe was removed as part of a surgical procedure for carcinoma of the maxillary sinus. In this eye as in most of the other human eyes the aqueous:serum potassium ratio was generally below 0.90 whereas the sodium was considerably higher.

The sodium ratios generally conform to those previously published by others. To our knowledge, however, this represents the first time that a potassium ratio in excess of 1.0 has been observed. Our observation of a potassium steady-state ratio of 1.03 in the rabbit is at variance with the value of 0.89 reported by Langham and Taylor[9] in a series of five animals. The disparity is largely due to a difference between their plasma value (5.35 mEq/1,000 gm. of water) and our serum value (4.27 mEq/1,000 gm. of water). We are unable to resolve the difference at the present time. Fairly low aqueous:plasma potassium steady-state ratios have been reported in other species.[10]

The results quite clearly indicate that there is a difference in rate of movement of

TABLE 2

SODIUM AND POTASSIUM CONCENTRATIONS OF THE SERUM AND AQUEOUS IN THE HUMAN

| Diagnosis | Serum | | Aqueous | | Ratio | |
|---|---|---|---|---|---|---|
| | mEq/1000 gm. Water | | mEq/1000 gm. Water | | Aqueous/Serum | |
| | Potassium | Sodium | Potassium | Sodium | Potassium | Sodium |
| 1. Normal Eye | 5.37 | 154.8 | 4.65 | 147.7 | 0.86 | 0.95 |
| 2. Senile Cataract | 5.16 | 146.7 | 4.20 | 142.8 | 0.81 | 0.97 |
| 3. Senile Cataract | 5.20* | 158.7* | 4.28 | 148.3 | 0.82 | 0.93 |
| 4. Senile Cataract | 5.29 | 154.8 | 4.48 | 145.5 | 0.85 | 0.94 |
| 5. Senile Cataract | 4.56 | 149.7 | 4.44 | 145.5 | 0.97 | 0.97 |
| 6. Aphakia | 5.08 | 161.1 | 4.88 | 151.7 | 0.96 | 0.94 |

* Arterial serum.

TABLE 3

STEADY-STATE RATIOS OF MAJOR IONS

| Animal | Sodium | Potassium | Bicarbonate | Chloride |
|---|---|---|---|---|
| Rabbit | 0.94 | 1.03 | 1.28[1] | 1.01[1] |
| Monkey | 0.97 | 0.95 | 0.77[1] | 1.10[1] |
| Cat | 0.97 | 0.86 | 1.27[1] | 1.04[1] |
| Human (one case) | 0.95 | 0.86 | 0.93[1] | 1.13[2] |

[1] Data taken from chart compiled by Davson and Luck.[5]
[2] Hodgson, T. H., J. Physiol., 94: 118 (1938).

cations into the aqueous, assuming of course, that steady-state ratios reflect a balance of rates of entrance and exit. It is immediately apparent that the results cannot be attributed to differential mobility of sodium and potassium. Actually measurements of the movement from plasma to aqueous of radioactive ions have indicated that the rate of movement of the two into the anterior chamber from the plasma is generally the same.[11]

As previously noted species variations of the anions have been reported. It is interesting to note that in a small series there is no correlation between the distribution of chloride and bicarbonate on the one hand and sodium and potassium on the other (table 3).

These results suggest that the distribution of potassium is actively controlled, although not necessarily actively transported. Accordingly, in a second series of experiments we have investigated the effect of Diamox on the potassium and sodium contents of the aqueous and serum. The studies were deemed particularly imperative because this drug may have a specific effect on potassium movement as judged by its influence on potassium excretion.[12] Previous work of others has indicated that the sodium concentration of the aqueous is not appreciably altered by Diamox.[13,14] A decreased aqueous potassium concentration has been observed.[13]

Following the injection of Diamox* there was a fairly marked decrease in the potassium concentration of the aqueous. In every instance the fall in potassium concentration could be attributed to a decrease in serum level so that the steady-state ratios remained essentially unaltered or actually increased (fig. 1). It is interesting to note that the 15-minute value did not show a preferential decrease in aqueous potassium. The result is thus not comparable to that of Langham and Lee[14] who found a decrease in aqueous bicarbonate but no change in plasma level at this time interval. The sodium content was not appreciably altered except at six hours when a slight fall in the serum level was observed (fig. 2). A decrease in serum potassium with no attendant change in serum sodium is a fairly typical finding when Diamox is administered.[15, 16]

A fairly high dosage of Diamox was required to induce this change in the serum.

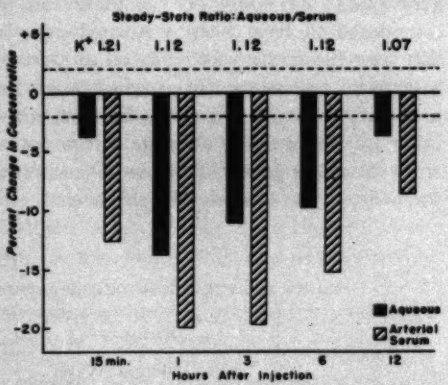


Fig. 1 (Harris, et al.). Influence of Diamox (50 mg./kg.) on serum and aqueous potassium concentrations. Calculations are made from measurements of the concentration of potassium in the aqueous and serum of the same animal before and after injection of the drug. The dotted line represents the average difference in aqueous potassium concentration between the two eyes of the same animal (1.97 percent). The data of the figure represent a total of 46 experiments.

* The Diamox was kindly furnished by Gordon Dempsey, M.D., Lederle Laboratories Division, American Cyanamid Company.

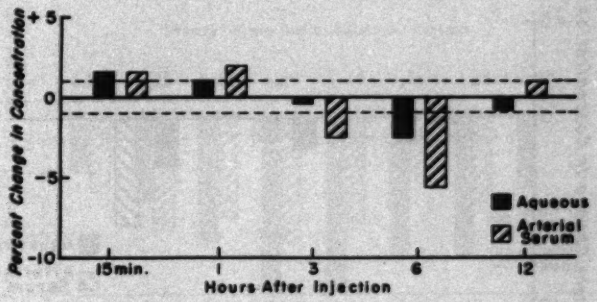


Fig. 2 (Harris, et al.). Influence of Diamox (50 mg./kg.) on serum and aqueous sodium concentrations. Calculations are made from measurements of the concentration of sodium in the aqueous and serum of the same animal before and after injection of the drug. The dotted line represents the average difference in aqueous sodium concentration between the two eyes of the same animal (1.07 percent). The data of the figure represent a total of 46 experiments.

Thus, making measurements at six hours, little change in potassium concentration was observed when 15 mg./kg. was given; the change was fairly appreciable when 30 mg./kg. was used (fig. 3). This is of interest since fairly marked alteration in bicarbonate of both plasma and aqueous was obtained by injecting only 15 mg./kg. of Diamox. Again little or no change in the sodium was observed in any of these concentrations (fig. 4).

It is, of course, to be noted that these cation measurements are calculated as percent change in concentration. The maximum change in potassium concentration observed was in the neighborhood of 1.0 mEq/1,000 gm. of water, not an impressive osmotic force. In no instance did the balance lower osmotic activity of the aqueous with respect to serum. The error of the sodium measurement, some 1.0+ percent, represents a higher osmotic force than the total (and significant) alterations in potassium.

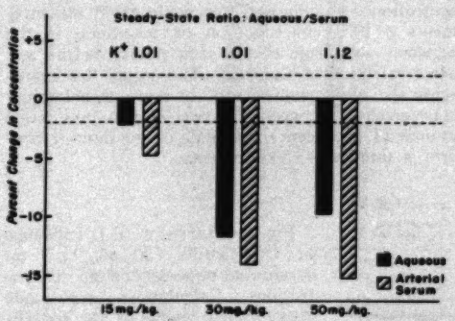


Fig. 3 (Harris, et al.). Change in serum and aqueous potassium concentrations six hours following intravenous injection of indicated amount of Diamox. Calculations are made from measurements of the concentration of potassium in the aqueous and serum of the same animal before and after injection of the drug. The dotted line represents the average difference in aqueous potassium concentration between the two eyes of the same animal (1.97 percent). The data of the figure represent a total of 29 experiments.

Dibenamine is a known ocular hypotensive agent which apparently inhibits aqueous formation.[17] We have accordingly measured the effect of this drug on the cation exchange. The results were similar to those obtained with Diamox. Dibenamine produced a marked drop in the potassium level of the serum and an attendant drop in the aqueous (fig. 5). In every instance the aqueous drop could be attributed to a fall in the serum, the steady-state ratios generally tending to rise because of the greater decrease in serum value. Sodium was essentially unaltered (fig. 6). A few years ago we also reported that Dibenamine reduced the total carbon dioxide content of the aqueous but demonstrated that this decrease generally followed an attendant drop in the plasma level.[17] With Diamox, however, we usually found a somewhat greater fall in the total carbon dioxide of the aqueous than in the plasma although plasma values changed considerably (fig. 7).* Generally, the steady-state ratio was not altered, however, and the results are thus similar to those recently reported by Langham and Lee.[14]

* These data were presented at the XVII International Congress of Ophthalmology in New York, 1954, but were not included in the published proceedings. It is published here since there has been some disparity between various reports on the effect of Diamox on bicarbonate.

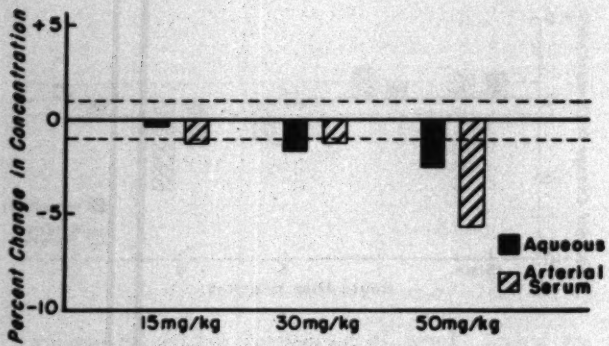


Fig. 4 (Harris, et al.). Change in serum and aqueous sodium concentrations six hours following intravenous injection of indicated amount of Diamox. Calculations are made from measurements of the concentration of sodium in the aqueous and serum of the same animal before and after injection of the drug. The dotted line represents the average difference in aqueous sodium concentration between the two eyes of the same animal (1.07 percent). The data of the figure represent a total of 29 experiments.

We have also studied two other diuretics.* One of these, dichlorphenamide, is stated to be approximately 30 times as potent an inhibitor of carbonic anhydrase on a mole for mole basis as is Diamox. Dichlorphenamide induces a decrease in the plasma bicarbonate and markedly inhibits the reabsorption of potassium in the kidney tubule, behaving in a manner similar to Diamox in this respect. Chlorothiazide, a weak carbonic anhydrase inhibitor, does not cause this preferential decrease in potassium reabsorption and in useful dosage little alters the bicarbonate levels.[18]

In our clinical studies we have found dichlorphenamide to be a very potent ocular hypotensive agent, whereas, at least in acute dosage (of up to 1,000 mg.), chlorothiazide

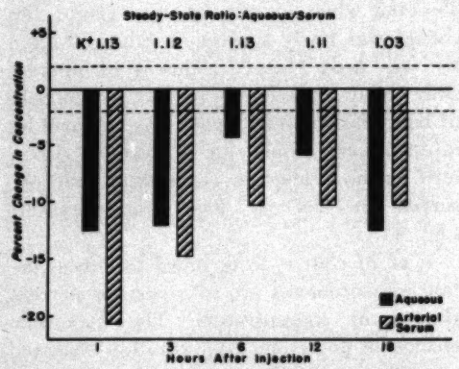


Fig. 5 (Harris, et al.). Influence of Dibenamine (20 mg./kg.) on serum and aqueous potassium concentrations. Calculations are made from measurements of the concentration of potassium in the aqueous and serum of the same animal before and after injection of the drug. The dotted line represents the average difference in aqueous potassium concentration between the two eyes of the same animal (1.97 percent). The data of the figure represent a total of 43 experiments.

* The chlorothiazide (Diuril) and dichlorphenamide were kindly furnished by John R. Beem, M.D., of Merck Sharp and Dohme.

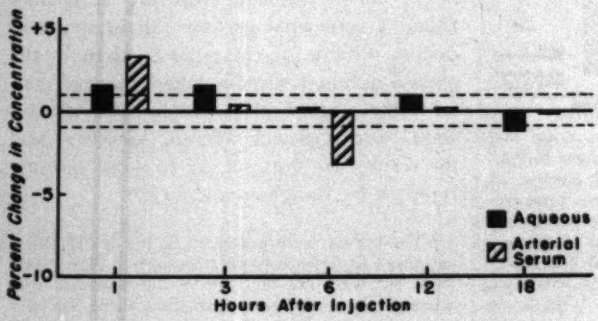


Fig. 6 (Harris, et al.). Influence of Dibenamine (20 mg./kg.) on serum and aqueous sodium concentrations. Calculations are made from measurements of the concentration of sodium in the aqueous and serum of the same animal before and after injection of the drug. The dotted line represents the average difference in aqueous sodium concentration between the two eyes of the same animal (1.07 percent). The data of the figure represent a total of 43 experiments.

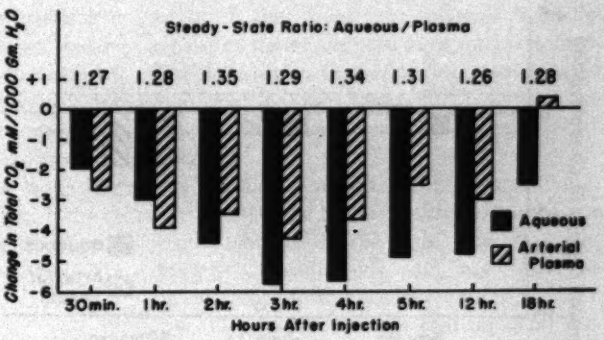


Fig. 7 (Harris, et al.). Change in total carbon dioxide concentration of plasma and aqueous following intravenous injection of Diamox (15 mg./kg.). Calculations are made from measurements of the concentration of carbon dioxide in the aqueous and serum of the same animal before and after injection of the drug. The data of the figure represent a total of 66 experiments.

appears to be without effect. Apparently, dichlorphenamide does not induce as rapid a drop in the intraocular pressure as does Diamox in the human but appears to be more effective than the latter in chronic dosage. Control of the ocular tension of certain patients has been established with dichlorphenamide when Diamox had previously failed.

The effect of these drugs on the cation content of the serum and aqueous gave the predicted results (fig. 8). Dichlorphenamide in low dosage produced a fairly marked decrease in the potassium concentration of the serum and a concomitant decrease in that of the aqueous. Chlorothiazide in the same dosage on the other hand had little effect on the potassium levels of either serum or aqueous. Neither drug appreciably altered the sodium concentrations (fig. 9). Five mg. per kg. of dichlorphenamide had approximately the same effect as 50 mg. per kg. of Diamox.

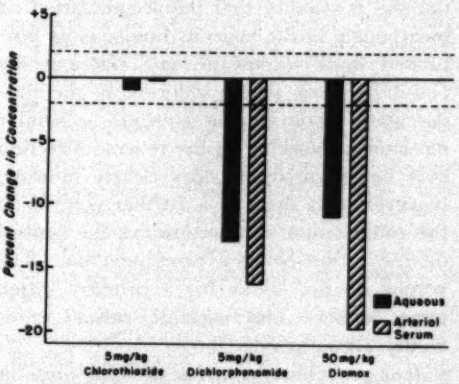


Fig. 8 (Harris, et al.). Change in potassium concentrations of serum and aqueous three hours following intravenous injection of indicated amount of drug. Calculations are made from measurements of the concentration of potassium in the aqueous and serum of the same animal before and after injection of the drug. The dotted line represents the average difference in aqueous potassium concentration between the two eyes of the same animal (1.97 percent). The data of the figure represent a total of 28 experiments.

## Discussion

It is interesting to note that those drugs which are ocular hypotensive in the human have two effects in common when administered to the rabbit. One, they generally reduce the bicarbonate concentration of the plasma and aqueous and, two, they produce a decrease in aqueous and serum potassium. A relationship between alteration in bicarbonate and potassium is a fairly common clinical experience and the parallel changes here reported are probably more than coincidental even though their significance is obscure. It is important to note, however, that the usual relationship is almost uniformly the reverse of that seen here, that is, generally a low bicarbonate is associated with a high potassium. This has been abundantly observed in both clinical and laboratory situations in which the bicarbonate is altered by a variety of means.[19-22] This inverse ratio is probably more a reflection of the pH than the bicarbonate level so that it

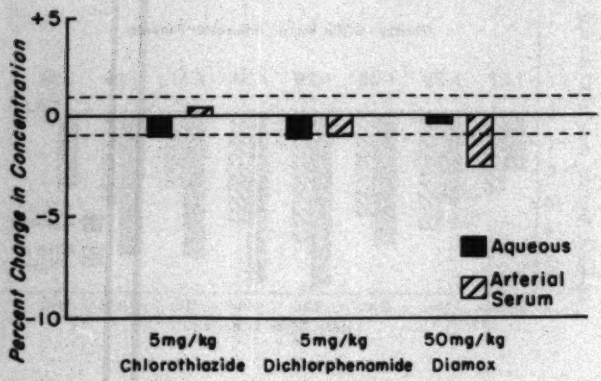


Fig. 9 (Harris, et al.). Change of sodium concentration of serum and aqueous three hours following intravenous injection of indicated amount of drug. Calculations are made from measurements of the concentration of sodium in the aqueous and serum of the same animal before and after injection of the drug. The dotted line represents the average difference in aqueous sodium concentration between the two eyes of the same animal (1.07 percent). The data of the figure represent a total of 28 experiments.

is perhaps more accurate to state that, except for the drug studies mentioned above, a high hydrogen ion concentration is associated with elevated potassium levels.[19, 20]

One may question the credibility of the conclusion that the hypotensive action of any one drug is due to carbonic hydrase inhibition. It is not unreasonable from these results to conclude tentatively that Dibenamine, an adrenolytic and sympathicolytic agent, and Diamox have a common site of action. At the same time the general truth that the most prevalent action of a drug (carbonic anhydrase inhibition in the case of Diamox) is responsible for its major systemic effect is reasonable. He that would propose a contrary view must shoulder the burden of proof.

In all likelihood the key to the action of these drugs may not be found in the alteration of potassium or bicarbonate distribution. Some other substance, may provide a better clue to their effects. This may be the hydrogen ion although such a statement is purely speculative. It is likely that both hydrogen and potassium enter the aqueous via a cellular route. The thought of Berliner, et al.[12] that the two compete for a common carrier in the cells of the kidney tubule is intriguing in the light of our observations reported here and those of Langham and Lee[14] that the pH of the aqueous (and plasma) falls following Diamox. An influence of carbonic anhydrase inhibitors on cation distribution in the lens[23] and certain other cells is well known although the effect may not be strictly due to carbonic anhydrase inhibition.[24]

In any event, the results here described clearly indicate that one cannot consider aqueous formation as due solely to the active transport of an anion, be it chloride or bicarbonate. This does not of necessity rule out the possibility that the accumulation of bicarbonate in the aqueous humor is as postulated by Friedenwald[2] nor rule out the possibility that where chloride is the predominate anion of the aqueous, a similar mechanism working in the reverse direction may be operative. It does clearly indicate, however, that there is a further control on the composition of the aqueous, the control on the cation being a prominent one. The results do not speak for a primary cation pump either. The variable cation ratios would argue against this.

It is probably erroneous, then, to consider aqueous secretion and aqueous flow as deriving from the active transfer of some particular substance the movement of which supposedly creates an aqueous hyperosmotic to plasma. Rather it is to be considered that the aqueous is secreted as a unit the composition of which is modified when the plasma levels presented to the ciliary epithelium vary. It seems reasonable to suppose that

the movement of some particular substance (as yet unknown, but probably not bicarbonate) may ultimately control the rate of aqueous formation. This substance may not of necessity be present in higher concentration in the aqueous than the plasma. Glucose, an essential nutrient to the lens, may be such a constituent. Dibenamine, for example, appears to reduce the rate of glucose transport from plasma to aqueous.[17]

## Summary

1. A species difference in the cation steady-state ratios (aqueous:serum) was observed. While the sodium ratio was fairly constant, the potassium ratio was quite variable, varying from 1.03 in the rabbit to 0.86 in the cat.

2. Administration of Diamox and Dibenamine to the rabbit was followed by a decrease in the serum and aqueous potassium. The decrease in the serum level was greater than that of the aqueous. There was no change in the sodium values following either drug.

3. Dichlorphenamide, a more potent carbonic anhydrase inhibitor than Diamox, gave results similar to the latter but in lower dosage. Chlorothiazide, did not alter the cation values in the dosage employed.

4. The results indicate that aqueous formation cannot be accounted for solely on the basis of primary active transfer of an anion or a cation.

5. A possible relationship of the movement of the hydrogen and potassium ions is discussed.

*3181 S. W. Sam Jackson Park Road (1).*

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## Discussion

Dr. Irving H. Leopold (Philadelphia): Dr. Harris, in his typically meticulous fashion, has studied the problem of the inorganic cations in the ocular fluids. You will recall that potassium is thought to be the important inorganic cation within the cell fluids, and that sodium is considered the important inorganic cation of the extracellular fluids, and that together both are important in regulating the osmotic pressure in both the cells and the extracellular fluid.

In addition, both of them take part in the enzyme systems having to do with carbohydrate metabolism.

Dr. Harris today has confirmed earlier impressions of others that sodium is probably the most important cation in the ocular fluids. His analysis of aqueous humor and serum have suggested that the ratio of potassium of the aqueous to that of the serum is greater than unity in the rabbit eye, and less than unity in the other species such as the cat, the monkey and man. Previous analyses of the rabbit eye have suggested that the ratio is lower than unity.

The observations in the human eye are stimulating, particularly when one considers the ratios obtained in eyes which possessed almost mature senile cataracts.

You will recall that in 1942 Salit, Swan and co-workers demonstrated that the cataractous lens had a definite decrease in potassium concentration. One may wonder what has happened to the potassium in the lens and why it is not reflected in the aqueous humor of these eyes.

In the four-year period of Dr. Harris' careful study, other investigators have been extremely interested in posterior chamber chemistry. Perhaps Dr. Harris would care to discuss why he did not choose to do posterior chamber analyses.

In addition to this, Dr. Harris noted, as Franceschetti had reported previously, that the potassium levels dropped in the aqueous humor following the administration of Diamox. Dr. Franceschetti did not report serum analyses such as Dr. Harris performed, and it is Dr. Harris' opinion that this drop in potassium is reflecting the change in the serum. It is not due specifically to any local effect of the drug on the eye as far as the inorganic cation potassium is concerned.

In addition, Dr. Harris has had an opportunity to study new carbonic anhydrase inhibitors such as chlorothiazide and dichlorphenamide. These agents were discussed in November 1956 at the Schoenberg Memorial Lecture, and, as Dr. Harris mentioned, chlorothiazide has very little effect on the intraocular pressure and also very little effect on the potassium or sodium ions. Dichlorphenamide, however, has a remarkable effect on lowering the pressure and produces the same chemical changes in the anterior chamber and blood as Diamox.

The results with dichlorphenamide, tried at the Wills Eye Hospital by Dr. Gonzalez in non-glaucomatous eyes and recorded over a period of three hours, are on the accompanying slide and show a drop in intraocular pressure.

The next slide shows the results in glaucomatous eyes following 100 and 200 mg. of oral dichlorphenamide. Here again it is demonstrated that dichlorphenamide, a very potent carbonic anhydrase inhibitor, will lower intraocular pressure in glaucomatous eyes.

I have enjoyed the privilege of discussing Dr. Harris's excellent paper.

Dr. John E. Harris (closing): Thank you very much, Dr. Leopold.

The previously published results on rabbit eyes do differ from ours. Other investigators have found a somewhat lower ratio than 1.0 for potassium. The difference generally lies in the plasma values. On the average ours are somewhat lower. Since rabbit cells are fairly fragile, I feel that the lower plasma (in our case serum) value is probably correct. I would re-emphasize, however, that the rabbit has a poor homeostatic mechanism. Fairly rapid fluctuations on potassium levels do occur.

Our human studies with the newer drugs con-

firm the observations of Dr. Leopold that dichlorphenamide is a remarkably potent hypertensive agent.

As has been pointed out, the mechanism by which the carbonic anhydrase inhibitors lower the intraocular pressure is not certain. The evidence seems to indicate that the aqueous is secreted in fairly constant composition with respect to plasma under all measured situations. This is reflected in the fact that the osmotic pressure of aqueous with respect to plasma remains fairly constant when the carbonic anhydrase inhibitors are used. However, it is still conceivable that the movement of one particular substance may be the key one. Our own attention has been directed to the cations, particularly potassium, in recent months. The possible relationship between the movement of hydrogen and potassium has been mentioned. A role of ammonia cannot be ignored either. This will require future studies.

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